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XLVIII MARCH–APRIL, 1956 No. 2

FACTORS AFFECTING GROWTH AND DIFFERENTIATION IN SIMPLE SLIME MOLDS [1, 2]

KENNETH B. RAPER

(WITH 7 FIGURES)

The Acrasieae, a group of simple slime molds, afford exceptional material for demonstrating physico-chemical control of structural differentiation. In fact, it is possible to demonstrate, if not to understand, the manifestations of such regulation at virtually every stage in their development. This results in substantial measure from their unique life cycle and their singular morphogenetic behavior.

The Acrasieae are believed to occupy a position near the divergence of the plant and animal kingdoms—presenting in their vegetative stage a free-living amoeboid condition and in their fruiting phase a progressive integration and differentiation of the amoeboid cells to form plant-like structures consisting of supportive tissue and reproductive elements, or spores. They are usually grouped with or near the Myxogastrales, although they differ from these more widely studied Myxomycetes in certain important particulars: they do not exhibit a swimming stage, nor do the myxamoebae fuse at any time to form a true plasmodium. For this latter reason, they are increasingly and quite appropriately referred to as cellular, or amoeboid slime molds (Bonner, **1952** and in press; Sussman and co-workers, **1953**, **1954**).

[1] Paper presented as part of a symposium, "Physicochemical Control of Differentiation in the Fungi," American Association for the Advancement of Science, Berkeley, Cal., Dec. 28, 1954.

[2] Previously unpublished research reported in this paper has been aided by grants from the National Institutes of Health and the National Science Foundation.

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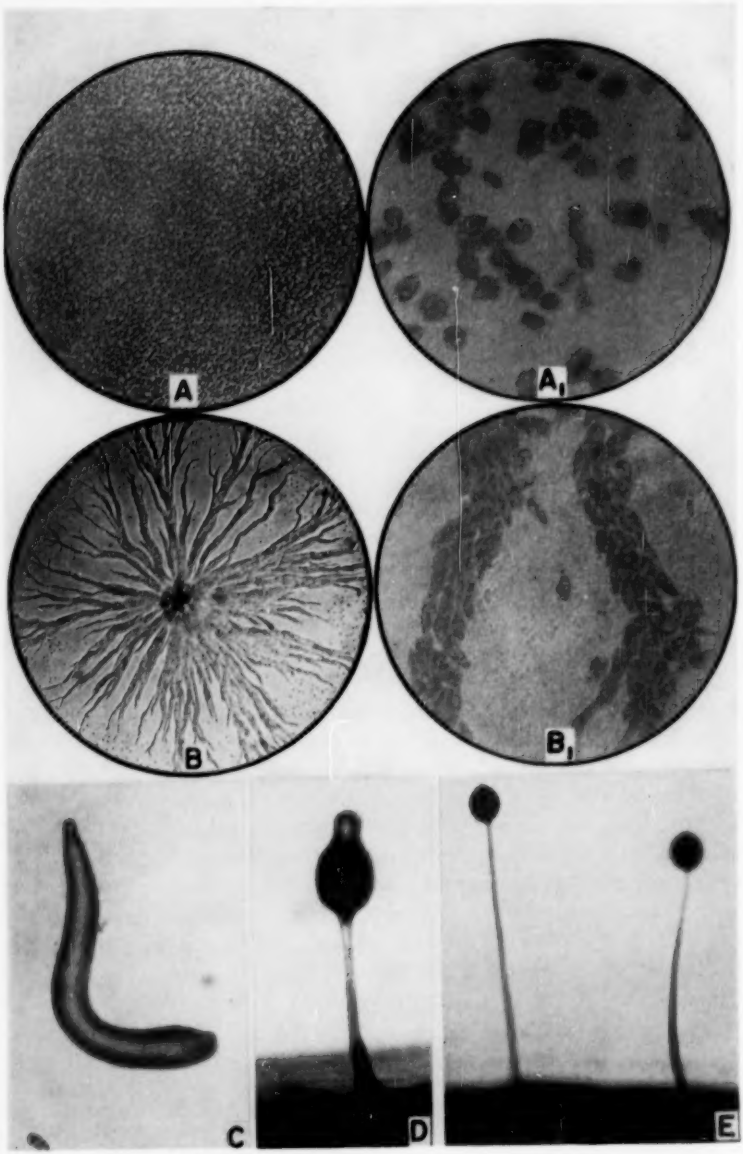

FIG. 1.

In the discussions that follow it will be necessary to use certain descriptive terms which are applicable to the different developmental stages exhibited by these slime molds. The cellular elements are amoeboid until the final stages of differentiation, hence are properly termed *myxamoebae,* although the contraction *amoebae* is commonly substituted. At the onset of the fruiting stage the myxamoebae collect into multicellular organizations termed *pseudoplasmodia.* During formation the pseudoplasmodium typically exhibits a radiate pattern of inflowing myxamoebic streams, hence is referred to as the *radiate* or *aggregating pseudoplasmodium,* or simply as the *aggregate.* In two species the pseudoplasmodium assumes the shape of a slug or cartridge and moves as a unit over the substratum for varying distances prior to fructification, hence is properly called a *migrating pseudoplasmodium.* The mature fruiting structure is termed a *sorocarp,* which in turn normally consists of an upright stalk, or *sorophore,* and a terminal spore mass, or *sorus,* composed of capsule-shaped reproductive cells, or *spores.* During fructification the mass of myxamoebae, largely undifferentiated, is termed the *sorogen.* The cycle is divided naturally into four stages, namely: growth or vegetation, aggregation, migration (if present), and fructification or culmination, as illustrated in FIG. 1.

GROWTH, OR VEGETATION

Whereas our present discussions are centered upon structural differentiation, these phenomena can seldom be dissociated from those of growth. In the cellular slime molds these developmental phases are dramatically separated in time, and commonly in space. Nevertheless, the conditions under which these microorganisms grow exert so marked an influence upon their subsequent development that I am constrained to recognize this in the title of my paper and to devote a limited amount of discussion thereto preparatory to any consideration of structural differentiation.

The Acrasieae are extremely sensitive to their environment throughout their entire life cycle. Growth is dependent upon the existence of favorable temperatures and humidities, and upon the presence of some

FIG. 1. Developmental stages in *Dictyostelium discoideum:* A. Vegetative stage with myxamoebae free-living and unoriented. A_1. Enlarged view of vegetative myxamoebae. B. Aggregating or radiate pseudoplasmodium showing conspicuous streams of inflowing myxamoebae. B_1. Detail of two small streams. C. Migrating pseudoplasmodium responding to 90° change in the direction of the light source. D. Developing sorocarp about midway in the process of culmination. E. Mature sorocarps. Scale variable.

suitable bacterial host upon which the myxamoebae can feed (Raper, **1937, 1939**). Light does not seem to affect materially the amount of myxamoebic growth, although it subsequently influences profoundly the behavior of the massed cells during the fruiting process. They are at all times strictly aerobic; hence growth and form development can occur only in the presence of free oxygen (Gregg, **1950**). They are unusually responsive to cultural conditions which can in the main be correlated with H-ion concentration. This relationship was investigated quite thoroughly many years ago for *Dictyostelium discoideum* Raper, and it was then reported that growth was precluded at pH 4.0 on the one hand and at pH 8.5 on the other. Between these extremes, growth increased steadily and, more significantly, subsequent development became more nearly optimal as the reaction approached a much narrower range from pH 6.0–6.5 (Raper, **1939**). With only limited differences, the same general behavior was observed in other species, including *Dictyostelium mucoroides* Brefeld, *D. purpureum* Olive, and *Polysphondylium violaceum* Brefeld (Raper, **1951**). More recently, Cohen (**1952**) has re-examined the interrelationship between growth and development of these slime molds and the reactions of the cultures in which they are grown. He concludes that acidity and alkalinity *per se* are not as limiting as I had inferred, and that at the higher reactions the presence of free ammonia constitutes the primary morphogenetic suppressant. His interpretation, if guardedly applied, may well prove to be the correct one. However, there can be little doubt that some of his conclusions were too all-embracing. For example, he reported that the genera *Guttulina* and *Guttulinopsis,* as studied by Olive (**1902**), may have represented only imperfectly formed sorocarps of some *Dictylostelium* or *Polysphondylium* that developed under very unfavorable conditions. The isolation of certain strains in our laboratory which under no circumstances differentiate beyond the stalkless or pseudo-stalked condition reported for these genera by Cienkowski (**1873**), Van Tieghem (**1880**), and Olive (**1901, 1902**) would seem to refute this argument effectively. We must always be cognizant that the very growth of these slime molds and the unfolding manifestations of their progressive differentiation continually reflect the degree of favorableness of each specific habitat—a condition which results from the composition of the substrate, the physiological activities of the associated bacteria, certain physical factors such as temperature and humidity, and the continual interplay of all of these factors one upon the other. One of the principal roadblocks currently limiting research with these slime molds is the lack of a wholly synthetic substrate upon which they can be readily grown in pure culture

free of bacterial cells, living or dead. Substantial progress toward this objective has been made by Sussman and Bradley (**1952, 1954**) but they are still short of realizing the ultimate goal since a limited amount of a bacterial protein must be included in the culture medium (ca. 2.5–5.0 mg/culture plate).

Despite these handicaps, we are able to grow many of the cellular slime molds quite satisfactorily in the laboratory, and in doing so we are able to profit from the many advantages they afford for investigating morphogenetic processes and cellular differentiation. Their outstanding advantage for this type of work stems from the sharp separation of their vegetative and fruiting stages. In no other group of organisms known to me can one study differentiation *per se* as completely apart from growth phenomena as in the Acrasieae. The life cycle may not be as simple as the sexless one generally accepted prior to Wilson's work of recent date (**1952, 1953**). On the other hand, it may not prove substantially more complex. Irrespective of the ultimate conclusion regarding sexuality, we now know from Wilson's study, and from concurrent investigations by Bonner and Frascella (**1952**), that nuclear divisions do occur after the myxamoebae enter the pseudoplasmodial stage and, as a consequence, there is an increase in cell numbers after the fruiting stage is initiated. We cannot longer accept the classic view that a certain number of building blocks (*i.e.* myxamoebae) are produced during the vegetative stage, and that the subsequent fruiting process consists simply in the orderly integration and differentiation of these same cells to form a sorocarp of a specific pattern and of dimensions determined only by the number of cells that aggregated to form the fruiting organization. Whereas adequate evidence of an increase in census growth, as Professor Harper would have designated it (**1929**), has now been found, we still have no evidence that any increase in protoplasmic mass occurs (*e.g.* as might be expressed by dry weight) after the myxamoebae aggregate to form the pseudoplasmodium. Viewed in this latter light, we can still, I believe, think of growth and structural differentiation as two quite distinct and successive phenomena.

To summarize, although vegetative growth and morphogenetic processes are separate from one another, the latter proceeds in a typical manner only when the cultural conditions are optimal for both. This close dependence can be obviated in some measure if the myxamoebae are grown in one type of culture and are then removed, washed, and transferred to a second and more favorable environment prior to initiation of the fruiting phase, for growth can occur under conditions which preclude normal fructification (Raper, **1939**). Even so the relationship

holds in the main, and the student of structural differentiation in these slime molds must at all times take cognizance of the conditions under which growth occurred as well as those under which he expects morphogenetic changes to unfold.

PSEUDOPLASMODIUM FORMATION, OR AGGREGATION

Let us assume that the slime molds are cultivated under optimal conditions, and that following a period of vegetative growth the myxamoebae come together and as a community of cells progress uninterruptedly through the various stages of fructification. These would seem to represent very simple processes, which of course they are when compared with growth and developmental phenomena in higher plants and animals. If we are anywhere to understand structural differentiation, it should be possible in the Acrasieae. But what do we actually know? What is responsible for the aggregation of large numbers of myxamoebae during pseudoplasmodium formation, and what are the physico-chemical controls operative here? What do we know concerning the organization of the mass of cells that comprise the pseudoplasmodium and how do the myxamoebae, collectively and individually, react to various stimuli? What subtle reactions are responsible for the continuing unitary organization of the pseudoplasmodium under some circumstances, and for its partial fragmentation under other conditions that outwardly seem equally favorable? For some of these questions we have partially affirmative answers; concerning others we have disappointingly little knowledge. I shall try to review briefly what information is available. I shall also attempt to focus our collective attention upon certain patterns of behavior concerning which our understanding is either non-existent or astonishingly superficial.

The manner in which previously independent myxamoebae flow together to form the pseudoplasmodium has engaged the attention of virtually very student of the Acrasieae (FIG. 1B). Brefeld, in his original paper (**1869**), reported and illustrated the aggregation of large numbers of cells preparatory to fructification. He misinterpreted, however, the correct nature of this stage and reported the myxamoebae as fusing to form a true plasmodium, an error subsequently corrected by Van Tieghem (**1880**). Potts (**1902**) was struck by the characteristic radiate pattern of the developing pseudoplasmodium and surmised that it arose in response to some chemotactic stimulus. Olive (**1902**) was even more strongly convinced and conducted limited experiments in the hope of proving this hypothesis. While he failed to demonstrate either attrac-

tion or repulsion of myxamoebae in the presence of added chemicals (*e.g.* malic acid), he made a very significant observation. He noted that the normal attractants had their origin in the myxamoebae and that they differed in different species, for fruiting structures of each type subsequently developed side by side apparently without intermixture when he planted white- and dark-spored species in the same localized area. In the years that followed, an explanation for aggregation was sought in other directions, and both Schuckmann (**1925**) and Harper (**1926**) concluded that the principal force was one of negative hydrotropy. Arndt (**1937**), in effect, returned to chemotaxis as the *modus operandi* in aggregation. He concluded that the stimulus responsible for aggregation arose within the myxamoebae themselves and varied in intensity in time and place. He showed that temporary movable centers of aggregation, termed *cumuli*, appeared and disappeared preparatory to the development of well-formed radiate pseudoplasmodia. With his time-lapse film of *D. mucoroides* he further demonstrated that the stimulus, or at least the response, varied in time, creating a wave-like motion within the mass of aggregating cells. This was subsequently demonstrated for *D. discoideum* in motion pictures made by John Bonner and by me. More recently, and here reported for the first time, I believe that we have evidence of the same phenomenon in a newly discovered species, *Dictyostelium polycephalum*,[3] for although we have not yet studied this *via* time-lapse photography, we do encounter developing pseudoplasmodia which show clearly discernible concentric bands of inflowing myxamoebae (FIG. 3C). It seems probable that this may represent a phenomenon common to all members of the group, and it remains to be shown how this fits together with other information concerning the mechanism of myxamoebic aggregation in these slime molds.

That the stimuli responsible for aggregation have their origin in the myxamoebae themselves and that these differ in different genera, and to a lesser degree in different species of the same genera, was evident from experiments which Doctor Thom and I reported in 1941. By cultivating myxamoebae of *D. discoideum* and *D. mucoroides* together on the same agar plate in association with the red-pigmented bacteria, *Serratia marcescens*, we were able to show that cells of the two species

[3] **Dictyostelium polycephalum** sp. nov.

Fructificio typice coremiformis, e parvis, saepe simplicibus, varians ad frequentiores sorocarpiorum compositorum fasciculos; sorocarpiis plerumque 375–650 μ altis, e sorophoris ad apicem disjunctis compositis; soris albis vel hyalinis, globosis, 45–90 μ in diam.; sporis ellipticis vel reniformibus, plerumque 6–7.5 μ longis, 3–3.5 μ latis.

initially contributed to the formation of a single radiate pseudoplasmodium, albeit the two kinds of individuals subsequently collected into different sorogens and built separate sorocarps typical of the two species. When *Polysphondylium violaceum* and a species of *Dictyostelium* (*e.g. D. discoideum*) were similarly cultivated, the two types of myxamoebae

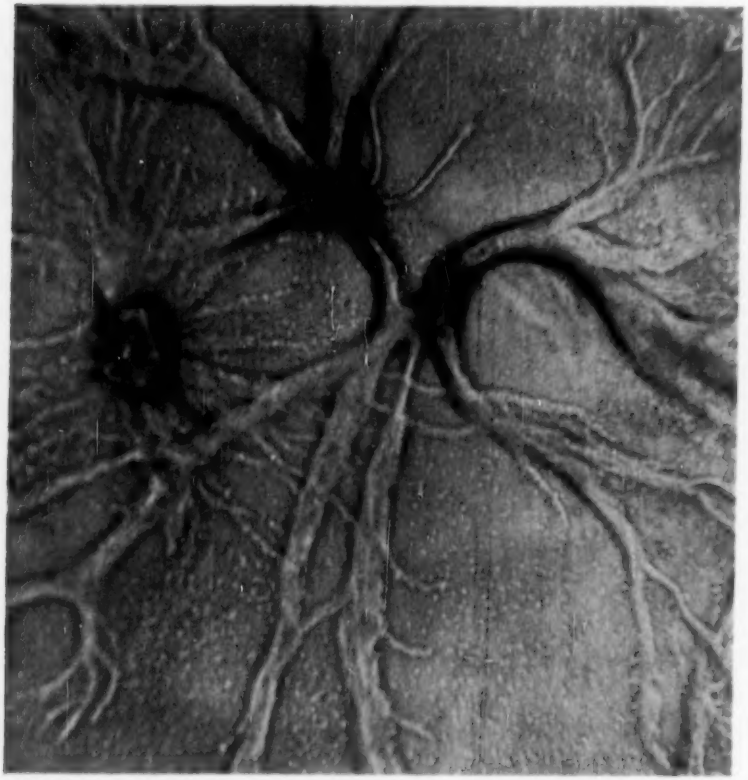

FIG. 2. Mixed culture of *Polysphondylium violaceum* (right) and *Dictyostelium discoideum* (left) showing separate and overlapping streams of myxamoebae of the two slime molds in an area where they grew as a mixed population. ×37.

aggregated to different centers from the outset, and the inflowing streams commonly overlapped (FIG. 2). Diffusion of the responsible stimuli through the culture medium was only inferred.

Clean-cut evidence for the production of a diffusible substance capable of exerting chemotactic attraction of unoriented myxamoebae was

obtained by Runyon in a simple experiment performed at Woods Hole in 1942. He laid a semi-permeable membrane (Visking) over an aggregating pseudoplasmodium and placed additional myxamoebae on top of this thin film. The latter accumulated directly above the pre-existing streams and centers of aggregation beneath the membrane. He concluded, therefore, that the aggregating cells produced a diffusible chemotactic substance of a molecular size and pattern which permitted it to pass through the dialyzing membrane. Apparently taking his cue from Runyon's work, Bonner (**1947**, *et seq.*) subsequently performed a series of elegant experiments from which we have gained most of our present information concerning the production and biological characteristics of the stimuli responsible for aggregation. He succeeded in demonstrating that the substance was produced not only by the centrally placed myxamoebae, but at decreasing concentrations outward in the inflowing streams as well. Additionally, he showed that the pseudoplasmodium continues to produce the substance throughout the period of migration and during progressive stages of sorocarp formation, the highest concentration being always in the apical area of the pseudoplasmodium or the rising sorogen. He further surmised that the same substance is responsible for the continuing polarity of the migrating mass and plays a most important role in regulating cellular differentiation during culmination. Finally, he gave the attractant a name, *Acrasin*—an always important step in lending stature to a new theory or an elusive substance, as this one unfortunately still remains.

Bonner's work has been done almost exclusively with *Dictyostelium discoideum* and the name "acrasin" has tended to become closely linked with this species. His original definition, however, presumed broader application, and today the term is used increasingly for a family of attractants, with appropriate descriptive modifiers being added to designate generic and specific types. Quite recently Shaffer, working first at Cambridge and subsequently at Princeton University, has made substantial progress toward characterizing the responsible agents in *Polysphondylium violaceum* and in four of the larger species of *Dictyostelium*. Whereas the title of his preliminary paper (**1953**) seemed a little too comprehensive, he succeeded in establishing certain important facts which provide valuable guideposts for future significant research. He demonstrated (1) that the frequent washings from established centers would induce chemotaxis of previously unoriented myxamoebae; (2) that the stimulatory substances were extremely labile, remaining active for only a very few minutes at room temperature but retaining their activity when frozen; (3) that the acrasin produced by species of

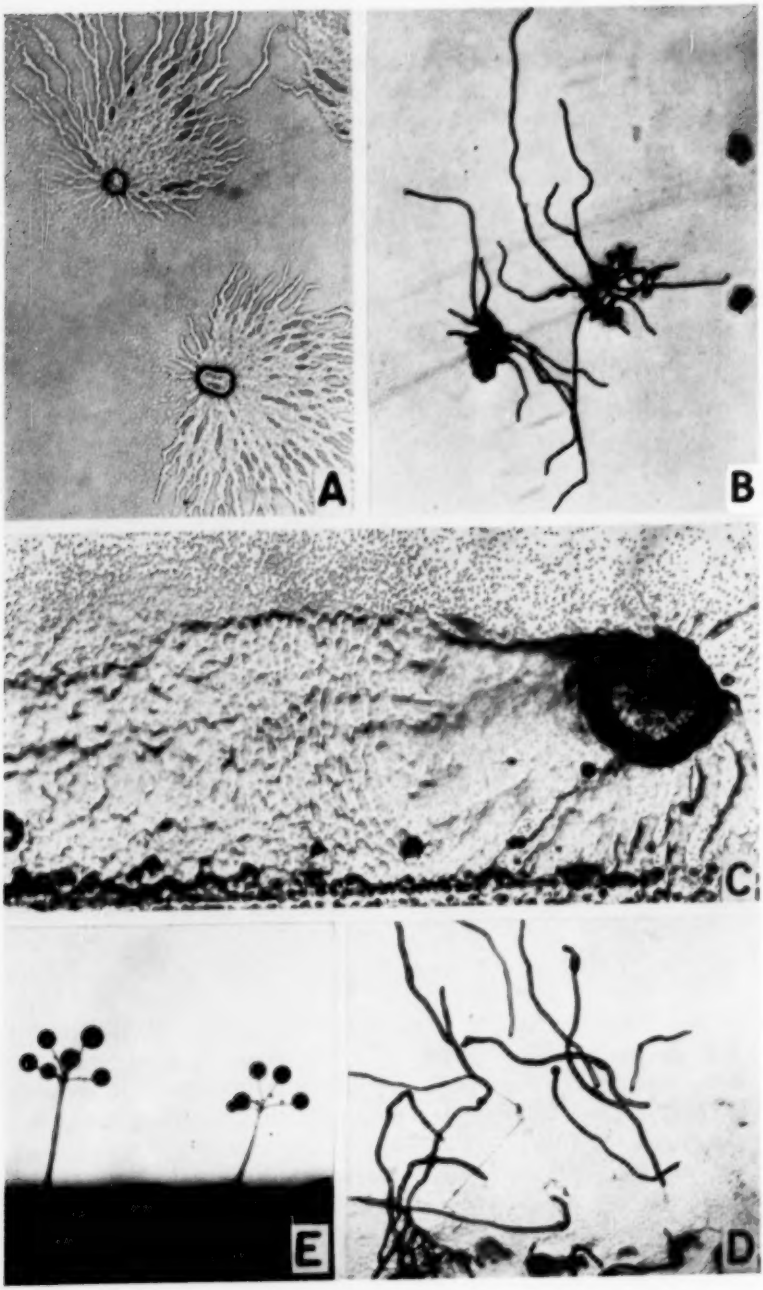

FIG. 3.

Dictyostelium was genus-specific, differing from that of *Polysphondylium;* and (4) that young *Polysphondylium* centers made only P-acrasin and attracted only myxamoebae of *Polysphondylium*, whereas older centers in *Polysphondylium* produced D-acrasin at the expense of P-acrasin. We anxiously await further reports of this work.

A reasonably coherent picture is beginning to emerge with regard to the phenomenon of aggregation in the larger and most-studied species. I am not so sure that observed facts fit the same pattern in the case of some of the more delicate species, particularly when we attempt to project acrasin-guidance as the principal regulatory agent in sorocarp formation to some of the more delicate species. In *Dictyostelium minutum* and *D. lacteum* it is the exception for an isolated sorocarp to arise from a single radiate pseudoplasmodium. In *Dictyostelium polycephalum* sp. nov. this rarely if ever occurs. Here, as a matter of fact, we see two stages at which fragmentation regularly occurs prior to culmination. A variable number of migrating pseudoplasmodia arise from the primary aggregate (FIG. 3B), each of which may give rise to from 1 to 10 sorocarps that are appressed basally but bear divergent sori at their apices (FIGS. 3D and 5D and E). (We shall return to this new and interesting species later.) In a second newly discovered slime mold, *Acytostelium leptosomum*,[4] an inordinately delicate form, behavior is equally interesting but quite different. In so far as I have been able to determine, single radiate pseudoplasmodia never give rise to single sorocarps. The myxamoebae characteristically come together into small radiate pseudoplasmodia (2.0 to 4.0 mm diam.) apparently typical of the group, but long before convergence is complete sorocarps begin to develop (FIG. 4, A). More often than not a dozen or more sorocarps will be formed from one primary aggregation, such fruiting structures developing intermittently at sites of greatest cell concentration along the inflowing streams (FIG. 4, C). Another striking phenomenon has been

[4] **Acytostelium leptosomum** gen. et sp. nov.

Sorocarpiis gregariis, summe teneribus e sorophoris acellularibus, plerumque 750–1500 × 1–2 μ, compositis, cum soris albis vel hyalinis, globosis, plerumque 30–50 μ in diam.; sporis sphaericis, plerumque 5–6.5 μ.

FIG. 3. *Dictyostelium polycephalum*. A. Aggregating pseudoplasmodia in a relatively thin culture. B. A later stage showing the origin of multiple migrating pseudoplasmodia from single aggregations. C. Aggregating pseudoplasmodium in a somewhat richer culture showing evidence of a wave-like movement of myxamoebae during aggregation. D. Edge of a culture showing numerous migrating pseudoplasmodia leaving the bacterial streak in which they developed. E. Clusters of mature sorocarps viewed from the side showing their characteristic coremiform pattern. A, B, D, and E, × 14; C, × 25.

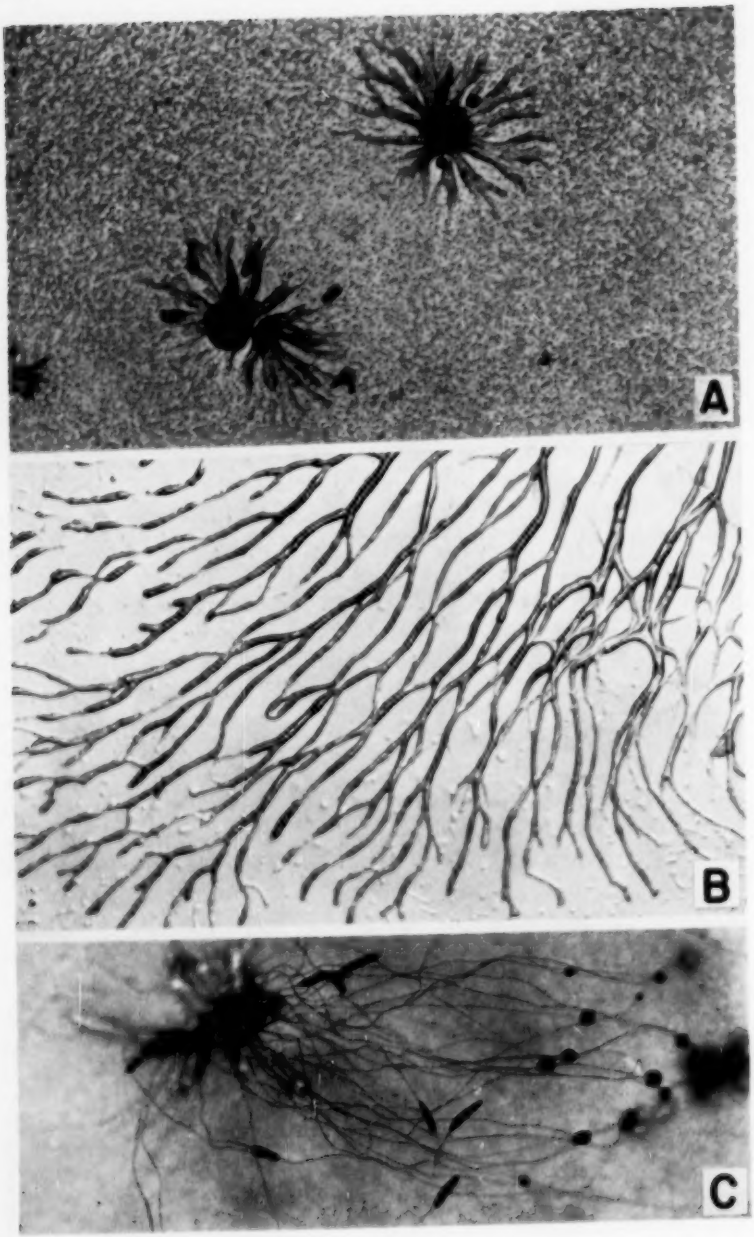

FIG. 4.

commonly observed in cultures of *Acytostelium*. After the initial aggregates (which are typically small and radiate in pattern) are formed, the feeding myxamoebae in an expanding front continue to consume the associated bacteria, while in the wake of this zone cells in broad expanses measuring up to 3.0–4.0 cm become oriented and collect into networks of interlacing streams possessing all the characteristics of developing pseudoplasmodia except that they are apparently devoid of centers that are needed for the completion of normal aggregation (FIG. 4, B). What explanation can we offer? Could it be that an Actyostelium-acrasin is produced and the myxamoebae are stimulated to line up in an orderly and characteristic fashion, but then the stimulus fades and they are left leaderless? If so, why do the streams persist, sometimes for days? Perhaps relatively vast fields of myxamoebae arrive almost simultaneously at a receptive stage, but the areas or points of acrasin production are so numerous or diffuse as to result in a virtual stalemate where no centers gain sufficient ascendency to command the myxamoebae in areas adjacent to them. I do not know the answers, but I suspect that these cultures tell us that we still have much to learn. Incidentally, this unique slime mold is especially interesting since it possesses an extremely thin, non-cellular stalk, a feature which we shall subsequently consider in some detail. These new slime molds are introduced here for the primary purpose of inquiring how strictly applicable to *them* are the presently unfolding concepts of acrasin regulation in aggregation, migration and culmination—concepts that are now predicated upon the behavior of the larger and more commonly isolated species.

There are two other recent investigations which are particularly germane to any discussion of myxamoebic aggregation. Starting with suspensions of washed vegetative myxamoebae of known concentration, Sussman and Noel (**1952**) found that the number of aggregation centers was maximal in *Dictyostelium discoideum* when unaggregated cells were added to the surface of washed-agar at a density of 200 cells/mm^2. In this species the ratio was about one center/2100 myxamoebae; whereas in *D. purpureum* the number of aggregates was much greater, being about one center/300 cells, while the optimal cell density for maximal aggregation was about 100 cells/mm^2. Based primarily upon mathe-

FIG. 4. *Acytostelium leptosomum*. A. Typical wheel-like aggregating pseudoplasmodia from which some sorocarps are beginning to develop, × 40. B. A portion of a larger area in which myxamoebae have collected into well-defined streams but without evident centers of aggregation, × 25. C. A cluster of sorocarps in various stages of development arising from a single pseudoplasmodium as illustrated in A above, × 25.

matical considerations, they concluded that each center probably owed its origin to a single physiologically specialized cell, termed an *initiator*. If cell density per unit area was too low, center formation might not occur for lack of an appropriately placed initiator; if it were above the optimal density (200 cells/mm² in *D. discoideum*), not all the potential initiators could form centers, for some would fall within the spheres of centers already in process of formation. An interesting extension of this work (Sussman, **1952**) showed that centers could be formed at lower densities of wild type cells (1:980) if these were augmented by cells of an aggregateless mutant to provide a substantial proportion of the responding cells. In contrast to the aggregateless mutant, which presumably possesses no initiator cells, Sussman has recently reported a mutant which shows one initiator per twenty-four responder cells, and produces correspondingly diminutive sorocarps (**1955**). Sussman does not think of the initiator cells in absolute terms, but recognizes that the attractiveness of a particular cell probably results from the relative responsiveness of the surrounding cell population as well as the strength of its initiating stimulus.

Recently, also, Charles Wilson (**1952, 1953**) has reported sexuality to occur in *Dictyostelium discoideum* and in a manner to profoundly affect aggregation and subsequent morphogenetic behavior. More than three decades ago, Skupienski (**1920**) had reported the fusion of myxamoebae as gametes in *D. mucoroides*, but his work had not been very seriously considered—perhaps because he had at the same time reported the formation of a true if somewhat transient plasmodium, and had presented unconfirmable claims of symbiosis between the ever-present bacteria and the slime molds (see Raper, **1937, 1939**). According to Wilson, who in the main confirms Skupienski's report—minus the plasmodium—the myxamoebae become gametes and fuse in pairs in the outer fringes of the aggregating pseudoplasmodia and then move as zygotes toward its enlarging center. Thus, he considers aggregation to represent a sexual phenomenon. Reduction divisions are reported to begin soon after the first zygotes form a center and to proceed as long as additional zygotes continue to join the aggregation. The zygote is said to be much larger than the haploid vegetating myxamoebae, and its nucleus is the only diploid nucleus in the life cycle. The haploid number of chromosomes is seven.

If we could satisfactorily integrate Wilson's observations with those of Bonner and Frascella (**1952, 1953**) we might conclude that the ability to produce acrasin, and hence to energize aggregation, represents a sexually induced capacity. Unfortunately this is not the case.

Bonner and co-workers failed to confirm Wilson's claim of gametic fusion; they did not observe that zygotes were much larger than vegetative myxamoebae; and they concluded that the chromosome number in *D. discoideum* is not seven but four, three of which consist of double arms. Finally, I should record that the Sussmans have thus far failed to confirm the existence of sexuality by means of genetical approaches. These points of difference should be resolved.

Whereas I would not suggest that the following factors are responsible for aggregation *per se*, we should recognize that certain environmental conditions exert in some way a marked influence upon the initiation of this process. Potts (**1902**), Oehler (**1922**), and Shuckman (**1924**) believed that the fruiting stage resulted from a food shortage, and in my own work (**1937**, *et seq.*) it has been clearly demonstrated that aggregates do not form until the available supply of suitable food bacteria has been consumed. Additionally, the onset of aggregation can be hastened from 2–4 hours by increasing the temperature of incubation, subjecting the cultures to decreased humidity, or exposing them to increased light (Raper, **1940**). Furthermore, the number of aggregates is increased, and their dimensions are reduced under each of these conditions. Such observations are not regarded as refuting in any way the theory of acrasin-induced aggregation. It is believed, however, that processes of cell maturation are probably speeded up in each case, so that an increased number of cells arrive earlier at the physiological stage where they can produce a chemical attractant (*e.g.* acrasin), hence initiate aggregation.

Hirshberg and Rusch (**1950**) have investigated the influence of various chemical compounds upon aggregation. Among these they found a group of azo dyes to be most active in limiting the phenomenon, whereas compounds including fluoride, malonate, sources of SH groups, antibiotics (penicillin and streptomycin), and anesthetics were inactive. The effect of 2,4-dinitrophenol upon early stages of differentiation was investigated in some detail (**1951**), with aggregation being precluded at concentrations above 10^{-4} M.

MIGRATION

For many years *Dictyostelium discoideum* was the only species of the Acrasieae known to exhibit a true migrating pseudoplasmodium (FIG. 1C). Recently we have on several occasions isolated from forest litter a second slime mold, *D. polycephalum*, which regularly exhibits a comparable developmental stage (FIGS. 3B and D, 5A). Since many

studies have been made with migrating structures in the former species by Bonner and me, I shall briefly review what is known regarding the morphology and behavior of these bodies, and then draw a contrast with the latter species, in so far as our present limited knowledge permits.

The myxamoebae of *D. discoideum* collect into a raised, peg-like structure as aggregation is completed. Then, unlike most other species where sorocarp formation is initiated immediately, this mass of myxamoebae, in the form of a slug or cartridge-shaped body, assumes a horizontal orientation and migrates as a unit for a variable distance before building a sorocarp. This migrating body is a most interesting structure and lends itself admirably to many types of experimentation (Raper, **1940**, *et seq.;* Bonner, **1944**, *et seq.*), and I believe that we can truthfully say that it accounts for much of the current interest in these simple slime molds. Structurally the migrating pseudoplasmodium is very simple, consisting entirely of a mass of closely integrated myxamoebae bounded by an envelope of slime, termed the *slime sheath,* which they secrete. We know relatively little regarding the intercellular organization of the myxamoebae which comprise the migrating pseudoplasmodium; we know less regarding the mechanics of movement whereby it achieves an orderly and unitary migration; we know almost nothing regarding the manner in which it receives and responds to external stimuli. We do know that it possesses an identity as a multicellular organization which is greater than the sum of its parts; we know that its internal organization is more important than the number or origin of the cells which comprise it; we know that it exhibits strong axial polarity; we know that it is extremely sensitive to its environment, and that such sensitivity and attendant responses are first expressed by the anterior tip; we know that this same anterior portion normally contributes to stalk formation, that its cells are characteristically oriented in a transverse direction, and that it shows a higher acrasin concentration than the more posterior regions; we know that all regions of the migrating body possess remarkable capacities for reorganization when its normal developmental course is interrupted or modified; we know that every portion of the pseudoplasmodium can form a sorocarp of a size proportional to its fractional mass; and, finally, we know that if the pseudoplasmodium is completely dismembered, its constituent myxamoebae retain the capacity to reaggregate into a variable number of smaller pseudoplasmodia, each of which will subsequently form a small but typical sorocarp.

Space does not permit full consideration of these matters. Furthermore, our knowledge of them is generally limited to observations on

behavior, with little or no insight into the physical or chemical bases of such conduct. I should like, therefore, to direct your attention to two comparatively recent studies by Bonner and co-workers wherein they have demonstrated remarkable sensitivity and responsiveness to well defined situations. Both have to do with factors affecting migration.

In the first of these (Bonner *et al.*, **1950**), it was demonstrated that the migrating pseudoplasmodia are extremely sensitive to temperature gradients. It had been known for a decade (Raper, **1940**) that such bodies moved toward the warmer side of a differentially incubated agar plate culture, but the true degree of sensitivity was in no wise appreciated. Using very sensitive thermocouples appropriately placed in a culture chamber, they were able to demonstrate that pseudoplasmodia responded to increased temperature where this gradient was 0.05° C/cm or, more dramatically expressed, where the difference in temprature at the two sides of a migrating body of 0.1 mm diam. was only 0.0005° C! They also investigated sensitivity to light and found that the pseudoplasmodium responded to all areas of the spectrum investigated within the range of 4800–6300 Å. It was then believed that the light response resulted not from a photochemical reaction but by differential heating, whereby the illuminated side would be slightly warmer than the darker side. Very recently Bonner and one of his students, Walter J. Gamble, have shown that there is an independent and equally photo-sensitive response governing orientation during migration.

In the second study, Slifkin and Bonner (**1952**) demonstrated that the extent of migration could be closely correlated with the concentration of salts and organic solutes present in the agar medium. The cultures were grown at room temperature on a buffered lactose-peptone medium with *Escherichia coli*. At the time of aggregation, disks of agar with developing pseudoplasmodia were removed, placed on cover glasses of equal diameter and then set upon the medium to be tested. Incubation was at 17° C thereafter. On washed agar the mean period of migration was 10.3 days (in one case 20 days). On media containing three selected organic non-electrolytes (dextrose, sucrose, and dl-alanine), migration continued for five or six days at low concentrations (< 0.1 M) and it became progressively less as concentrations were increased. Three selected salts (NaCl, $CaCl_2$, and Na_2HPO_4) were likewise studied and these were found to follow the same general pattern but to be 4–6 times more effective in limiting migration. It would be interesting to know whether somewhat higher incubation temperatures would have yielded comparable results. Nevertheless, the work clearly showed some previously unappreciated and still unknown factor as strongly affecting

migration. The results could not, they said, be entirely explained as an osmotic phenomenon.

As in the case of aggregation, pseudoplasmodial migration is strongly influenced by the physical environment. Migration can usually be precluded if a culture is moved from a temperature of 20–24° C to one of 28° C at the time of aggregation, in which case the pseudoplasmodium never leaves the site of formation but proceeds immediately to form a sorocarp *in situ*. Similarly, a migrating pseudoplasmodium will cease forward movement and initiate sorocarp formation at once when exposed to a similar rise in temperature. Conversely, if a culture is incubated or placed at 15–18° C, both the period and distance of migration will be substantially lengthened. Decreased humidity acts in the same manner as increased temperature, although quantitative data are not available. Light is most pronounced in its effect upon migration, the pseudoplasmodium regularly moving in the direction of the light source. Comparable responses are readily observed in *D. mucoroides*, *D. purpureum*, and *Polysphondylium violaceum*, except in these species the sorocarps are built toward the light, often resulting in structures with sorophores measuring several centimeters in length (see Raper, **1940**, *et seq.* for details). Obviously these physical forces influence in some undisclosed way the regulatory mechanisms governing migration and sorocarp formation. It is thought that they probably accelerate or retard important physico-chemical changes within the migrating body or the sorogen, respectively. It is not presumed that they act directly to effect the observed differences in behavior.

Now let us turn our attention briefly to *D. polycephalum*. Although this species possesses in common with *D. discoideum* the capacity to produce migrating pseudoplasmodia, these structures are quite unlike in the two species (cf. FIGS. 1, C, 3, D, 5, A), differing rather markedly in form, in behavior, and in the sorocarps which arise from them (cf. FIGS. 1, E and 3, D). In contrast to *D. discoideum*, where a radiate pseudoplasmodium typically gives rise to a single cartridge-shaped migrating body, the developing pseudoplasmodium of *D. polycephalum* regularly gives rise to a number of long, very thin, and variously contorted structures (FIG. 3, B). While rates of movement have not been determined accurately, it is obvious that the latter move more slowly than those of *D. discoideum*. They show only a limited and seemingly inconsistent response to light, and no investigations of temperature as a directional stimulus have yet been made. The anterior end is bluntly pointed as in *D. discoideum*, but the form of the migrating body is much more variable, and one gains the impression that it is less highly organ-

ized and much more fragile. The bounding membrane is obviously thinner, and it is virtually impossible to manipulate the pseudoplasmodia as in grafting experiments of the type so successfully carried out with *D. discoideum*. Locomotion of the pseudoplasmodium in *D. polycephalum* is even less understandable than in the older species. It is not uncommon to see moving bodies where only the posterior 1/10 is in contact with the substratum, or again to see a contorted body several millimeters long touching the substratum at only two or three places and with not more than 1/20 of its total length in contact with the agar. Weirdest of all is to see such a pseudoplasmodium moving through the aerial growth of a mucor and touching only two or three hyphae throughout its entire length. I cannot say that we yet have any tangible idea as to how a pseudoplasmodium moves, but it is obvious that nothing approachng uninterrupted contact of the moving body with a fixed surface is required.

Under optimal, but as yet poorly defined cultural conditions, the pseudoplasmodium comes to rest, often after several days, and completes the fructifying process, typically giving rise to multiple sorocarps (see p. 192). Under suboptimal conditions many migrating structures never reach this stage; others build simple sorocarps consisting of an erect, comparatively short sorophore terminated by a small globose sorus not unlike that seen in *D. minutum* Raper (**1941**).

CULMINATION

Our first insight into the unique fruiting process of the Acrasieae was provided by Van Tieghem three-quarters of a century ago (**1880**). He observed that the myxamoebae did not form a true plasmodium as Brefeld had originally reported (**1869**), and that the ensuing fructifications were constructed from aggregated but unfused myxamoebae. Subsequent workers added details. Olive's account (**1902**) is fairly representative of these, and it may be briefly summarized: Toward the close of aggregation certain centrally placed myxamoebae become vacuolate and as the central pseudoplasmodial mass enlarges by the continued aggregation of myxamoebae, other cells directly above the primary ones become vacuolate and take up the water that is being lost by the surrounding myxamoebae. In this way a column of conspicuously vacuolate cells is built up, and, as it lengthens, the main body of myxamoebae continues to ascend the column thus formed. When stalk formation has ceased and the diminishing mass of myxamoebae has reached its terminus, the then remaining cells become transformed into capsule-shaped

spores. He noted that the dimensions of the fructification were roughly proportional to the sporogenous mass and that the diameter of a given stalk decreased progressively the higher it rose above the substratum. The column of vacuolated stalk cells was reported to be surrounded by a sharply defined mucous sheath deposited by the ascending myxamoebae.

Professor Harper gave further attention to sorocarp formation in *Dictyostelium mucoroides* (**1926**) and *Polysphondylium violaceum* (**1929**), two of the species studied by Olive, and interpreted the process as essentially an architectonic one wherein free motile cells differentiated into either spores or enlarged vacuolate cells of supportive tissue depending upon such factors as position and pressures within the fructifying mass, or sorogen. He discussed at some length the so-called slime sheath that envelopes the completed sorophore, but he could not decide whether this was formed by the cells within the sorophore or by those surrounding it. In any case, he concluded that since it consisted of inert secreted substance it could only represent the product of individual protoplasts adjusting themselves to changing stimuli to maintain the correct taper of the sorophore. Like Olive and all earlier students of the group, he believed that the mass of myxamoebae ascended the rising sorophore through the coordinated, but nevertheless free amoeboid movements of each constituent cell. There was no suggestion by either Olive or Harper that the myxamoebae comprising particular areas of the pseudoplasmodium might normally contribute to the production of particular parts of the completed sorocarp.

The first evidence that this might be true was obtained from grafting and dissection experiments with *D. discoideum* (Raper, **1940**). By grafting red anterior tips onto white pseudoplasmodia it was possible to show that the anterior 1/4–1/5 normally produced the stalk, and the same conclusion was supported by experiments where the anterior portion was excised and induced to form a fruiting structure immediately, such a structure consisting essentially of a disproportionally large sorophore. These observations were subsequently confirmed by Bonner (**1944**), who, additionally, demonstrated that the stalk-forming cells showed a distinctive transverse orientation even in the migrating stage. He was thus able to show that the migrating body consisted of two distinct physiologically and functionally different parts, an anterior portion (roughly one-quarter) consisting of pre-stalk cells and a larger posterior portion comprised of pre-spore cells. He has further demonstrated this presumptive separation by vitally staining pseudoplasmodia and young sorocarps with nile blue sulfate (**1952**); the anterior or apical portion remains colored whereas the posterior portion blanches the dye and becomes virtually colorless.

Two years ago Miss Fennell and I (**1952**) analyzed in some detail the fruiting process in *D. discoideum*, and while we cannot claim to have revealed underlying causes, or the *modus operandi* of subtle physicochemical changes believed to regulate progressive stages in culmination, we do feel that we recorded with reasonable exactness the outward expressions of these. We were able to establish that stalk formation is initiated in the young sorogen by the deposition of a critically positioned and properly proportioned cell-free cellulosic membrane which seemingly entraps the cells that later become vacuolate and contribute to sorophore construction. This membrane, termed the *sorophore sheath*, is extended upward and is continuously formed well in advance of actual cell differentiation. Either directly or indirectly, it is thought to account for the principal lifting force responsible for the ascendance of the myxamoebae, at least during the later stages of sorocarp formation. We could not determine the origin of the sheath but recognized that it must of necessity be formed by the diminishing group of transversely oriented pre-stalk myxamoebae which comprise the apical papilla, or by the entrapped and unoriented cells within its terminal formative area. A secondary lifting force, physical in origin, of approximately equal but periodically greater magnitude was postulated to arise from the cohesive properties of the rising sorogen. Withal we were led to conclude that the phenomenon of stalk formation and the attendant differentiation of cells within and without the sorophore sheath represented, in some way, a response to forces and stimuli which had their origin in the unitary organization of the fruiting mass, rather than the individual reactions of the myxamoebae as free-moving cells as Harper had previously reported. In a recent paper, Bonner *et al.* (in press) show that carbohydrate of unstated character is concentrated in the proximal ends of the horizontally oriented cells adjacent to the site of sorophore sheath formation in the apical papilla. He points out that these, in effect, act as a transient secretory epithelium. The identity of its cellular components is continually changing, of course, as individual myxamoebae make their contribution to sheath extension shortly before they are pushed into the lengthening tube, and thereafter become vacuolate structural elements of the sorophore. Alkaline phosphatase activity was also followed and this was found to be most intense in the anterior area of the migrating pseudoplasmodium, and more particularly in the apical portion of the developing sorocarp.

Oxygen utilization at different stages of the developmental cycle of *D. discoideum* has been investigated by Gregg (**1950**). Using microrespirometers of original design, he showed that O_2 consumption increased markedly at the change from the vegetative to the migrating

stage, then remained constant until about the mid-point of culmination, when a gradual decline set in and continued until differentiation was complete and respiration ceased. This decline was interpreted as resulting from the increasing number of cells that were being differentiated either into spores or stalk cells and hence represented resting stages. It was noticed that larger structures developed more rapidly than small ones but that O_2 consumption per unit protoplasmic mass was the same in structures of all sizes. Bonner and Eldridge (**1945**) had previously discussed similar differences in rate of increase in height of sorocarps of different sizes, and had attributed these to the greater cohesive force resulting from an increased surface to volume ratio in small bodies. More work was required per unit mass, to effect a given linear increase in the latter, and this could be achieved only with increased time. A comparable phenomenon involving different rates of development in sea urchin eggs had been elucidated earlier by Tyler (**1942**) and attributed to the action of the principle of similitude. The same was thought to apply to *Dictyostelium*.

We know something of the changes which occur in fructification; we are now beginning to obtain information on how these changes may be effected; but we still know virtually nothing as to why they occur. What determines the division of labor among the constituent myxamoebae so that during culmination some cells are privileged to form spores while others are doomed to self-sacrifice in constructing the sterile supportive stalk? What subtle reactions and balances occur within the cellular mass that account for the specific patterns and consistent proportionalities of the completed sorocarps in different members of the group? We should like to know. Bonner (**1949**, *et seq.*) has shown that acrasin continues to be produced during culmination in *D. discoideum*, and that in contrast to earlier stages (*e.g.* migration) where it is formed in increasing concentration from the posterior end to the apex, it is in fact produced only in the contracted apical portion of the developing sorocarp. The steep gradient which results is thought to exert a profound effect upon fashioning the pattern of the rising sorogen and, by inference, in regulating the differentiation of its constituent cells.

Compared to certain other species, the process of culmination in *D. discoideum* seems to be quite straightforward. If produced under optimal circumstances and subsequently undisturbed, each aggregating pseudoplasmodium typically produces a single sorocarp of specific pattern and of appropriate dimensions. Elsewhere such is often not the case, and in certain species, it seldom if ever occurs. In *D. mucoroides*

and *D. purpureum* the situation is not too different, for single sorocarps generally arise from single aggregates, and under uniform environmental conditions these are reasonably consistent in form and pattern. Nevertheless, mature sorocarps often show extreme variability in the length of their sorophores, even from aggregates of approximately equal original mass. In these species, also, the young sorogen is extremely sensitive to its environment and this is strongly reflected in the length to which the sorophore is formed before the sorogen assumes a vertical orientation, after which culmination proceeds in much the same manner as in *D. discoideum*. It is believed very significant, as Bonner has shown (in press), that whereas the area of presumptive stalk cells in these species remains small during periods of extended stalk formation, this area is continually augmented from the reservoir of presumptive spore cells. Thus a continuing readjustment in the ratio of presumptive stalk *vs.* spore forming myxamoebae can and does occur, whereas in *D. discoideum* such demarcation is established early and normally remains essentially unchanged unless major modifications are imposed upon the fruiting organization.

In *Dictyostelium minutum* and particularly in *D. lacteum*, species characterized by diminutive sorocarps, aggregating pseudoplasmodia generally give rise to multiple sorocarps of delicate proportions. Whereas culmination has not been investigated in either of these species in detail, it is believed to proceed in approximately the same manner as in the larger forms once sorocarp formation has been initiated. This does not, of course, provide an explanation for the fact that single aggregates normally produce several sorocarps. Thinking in terms of acrasin production, could it be that these species produce relatively weak chemotactic stimuli which can attract the myxamoebae initially but are not sufficiently intense to hold together and continue to regulate the activities of the entire population? Or alternatively, could we postulate a diminution in acrasin production at the close of aggregation?

The genus *Polysphondylium* presents a further complication. Here we commonly encounter the development of more than one sorocarp from a single aggregate, and this is especially true in *P. pallidum*. However, there is an even greater hurdle to overcome. As the sorogen is raised in the air, initially as in *D. mucoroides* or *D. purpureum*, posteriorly located fractions periodically lag behind, become vertically transected, and give rise to whorls of lateral branches, each of which resembles a diminutive sorocarp that is anchored to the main sorophore axis. Can we here assume a periodic diminution in acrasin production whereby a fraction is cast off of the size required to again bring into

balance the strength of the apically produced regulating stimulus and the mass of myxamoebae to be regulated? We could, I think, without difficulty envision the formation of the lateral branches, for although the acrasin concentration in the posterior-most fraction would presumably be low, it might in such fractions still be relatively high in relation to the mass of cells to be influenced by it. How to account for the vertical transection of the mass to form a number of equally spaced secondary sorogens is not at once obvious. Alternatively, we may be dealing with correlation rather than cause, and I cannot but wonder if the wave motions seen in the aggregating stage may not carry through and in some way effect the periodic lag of posteriorly placed groups of myxamoebae. Unfortunately this also provides no clue as to why the mass becomes divided once it loses contact with the larger advancing frontal mass. Perhaps we should record that the cast-off masses typically decrease in size as the main body of cells advances, hence normally contribute to the formation of a radially and metamerically symmetrical sorocarp which in its basic pattern is, as Professor Harper stated (**1929**), strongly suggestive of a miniature pine tree. This pattern is particularly striking in the completed sorocarps of *Polysphondylium pallidum*, a white-spored species with which Professor Harper apparently never worked.

More than two decades ago, Harper (**1932**) made a detailed analysis of the sorocarps of *P. violaceum* when incubated in light and in darkness. He found that the sorocarps were less numerous and averaged larger, with more whorls of branches, in dark-incubated cultures. I would venture the guess that comparable responses could be obtained with lowered temperatures as well.

In species of *Polysphondylium*, even more than in *Dictyostelium discoideum*, the attainment of a properly proportioned structure would of necessity seem to represent an expression of the overall organization which pervades the fruiting mass.

Still a different pattern of sorocarp formation characterizes *Dictyostelium polycephalum*, and concerning this we have virtually no information regarding the presumptive alignment of myxamoebae preparatory to culmination. I have previously stated that the species forms a migrating pseudoplasmodium, and that this structure typically produces more than one sorocarp (FIGS. 5, A–E). It is of interest to examine the manner in which this takes place. After a variable period of migration extending up to several days and often covering a distance of 3–4 cm, the pseudoplasmodium comes to rest, collects into a rounded mass, and then develops from its upper surface a number of conspicuous

papillae. Sorophore formation is initiated almost immediately in the area beneath each of these, and as the developing stalks elongate the mass of cells becomes vertically divided into a like number of separate but essentially parallel fractions, or sorogens (FIG. 5, B). The stalks stand in close proximity at their bases, each being somewhat expanded where it contacts the substratum (FIGS. 5, C and D), and continue to be constructed parallel to approximately three-fourths their ultimate length, at which level they diverge sharply with each bearing at its terminus a small rounded sorus (FIGS. 5, D and E). Thus we have in this species a coremiform *Dictyostelium*. Viewed under the low-power binocular one gains the impression usually of a single, albeit somewhat heavy stalk which branches terminally; viewed under higher magnifications the true character of the structure is revealed, for each sorophore can usually be traced without difficulty from base to terminal sorus. The degree of adherence varies, and in many structures the separate stalks stand clearly apart near the substratum but are closely appressed for some distance below the point of divergence. While careful studies remain to be made, it would seem that spore maturation begins to occur at approximately the time the stalks build apart from each other. Whereas the building of each separate sorophore is believed to follow essentially the same pattern as in other Dictyostelia, we have not as yet observed the characteristic stage where the ripening sorogen approaches a sphere and is terminated by a much-constricted apical papilla in which the final phase of sorophore differentiation occurs. The spores are smooth-walled and capsule-shaped as in most members of the group; the structure of the sorophore, commonly consisting of a single row of superimposed cells bounded by a thin and continuous sorophore sheath, is basically similar to but often less regular than that of *D. minutum*. Single sorocarps, each consisting of a simple upright stalk and sorus, can be seen in any fructifying culture, and structures of this type may represent the only type of fructification present in plates where conditions are not optimal. In other cultures, and resulting from circumstances still unresolved, "compound sorocarps" are regularly produced, the number of discrete fruiting structures ranging in number from two to ten and with 4 to 7 being commonplace in a single cluster. The species is of special interest since it presents a structural pattern not previously encountered, although it should be recalled that a coremiform stalk was reported for *Acrasis* by Van Tieghem (**1880**). In this latter genus the spores were spherical and slightly roughened and formed a catenoid chain rather than collecting as an enslimed mass. It is not suggested that the two forms are especially close phylogenetically.

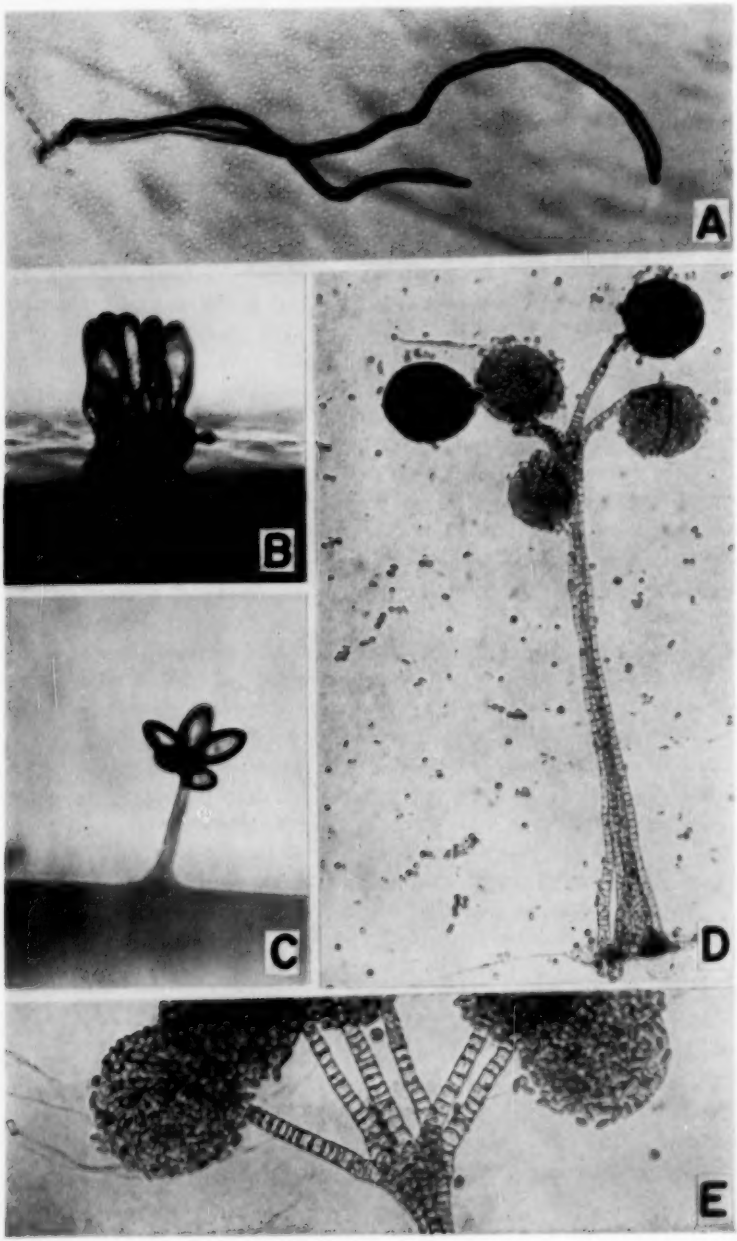

FIG. 5.

Chief interest in *Dictyostelium polycephalum* arises from the dual characteristics of a migrating pseudoplasmodium and compound fructification bearing a variable number of divergent sori (FIG. 5). How can we account for the fact that a pseudoplasmodium characteristically produces multiple sorocarps following a period of migration? We know that the migrating pseudoplasmodium of *D. discoideum* commonly divides dichotomously, thus giving rise to two smaller but otherwise normal migrating bodies, each of which subsequently produces a typical sorocarp. We know that two migrating bodies of different origin may fuse and later build a single typical sorocarp. But in both of these cases, once the pseudoplasmodium has come to rest it produces only *one* sorocarp unless it is radically disturbed. The normal course of events in *D. polycephalum* is thus strikingly different. What explanation can we suggest? And why do the at first neighborly sorogens build apart from one another once spore formation has begun? Returning to some possible chemical substance, which for convenience we might call polycephalum-acrasin, could it be that this is produced at one level during aggregation, at a lower level when the few to several migrating pseudoplasmodia emerge from the aggregate, and at a still lower level when each of these bodies ceases migration preparatory to sorocarp formation? Or conversely, is an increasingly higher level required to carry through the regulatory function required for morphogenesis at each successive stage, and in the absence of such does the mass twice fragment iself in order to reduce the functional masses to an acrasin level which is attainable? I certainly do not know. Acrasin as we now know it may even play a minor role. But by posing such questions, ideas for experiments may occur to us, and through experimentation we may in time find explanations. Turning now to the developing cluster of sibling sorocarps, could it be that these begin to build apart at a critical stage when some hypothetical "anti-acrasin," or repellant substance, makes its appearance? This too is wildest speculation. But something happens, and groups of amoeboid cells acting as organizational units respond.

I cannot leave *Dictyostelium polycephalum* without citing an inter-

FIG. 5. *Dictyostelium polycephalum*. A. Two typical migrating pseudoplasmodia, × 25. B. A cluster of sorocarps just starting to develop from a single migrating pseudoplasmodium, × 70. C. A more advanced stage in a different cluster, × 70. D. A mature cluster of sorocarps showing the coremiform character of the over-all structure and the individuality of the constituent sorocarps, × 170. E. A somewhat enlarged view of the terminal portion of a different coremiform structure, × 320.

esting cultural situation where physico-chemical factors of unknown nature apparently exert an effect upon sorocarp formation. This species was isolated originally by Miss Fennell and me from forest soil, using the routine device of planting it at the intersection of crossed streaks of *Escherichia coli* on hay infusion agar plates. The slime mold grew luxuriantly and some migrating pseudoplasmodia were produced, but it fruited very sparingly or not at all. Yet, in the grossly contaminated source plates containing various fungi and assorted bacteria it fruited reasonably well. In part deliberately and in part by chance, we soon found that it would fruit in 3-membered cultures, and we now grow it most satisfactorily in cultures containing the slime mold, *Aerobacter* sp. (Singh's strain, **1947**), and the yeast-like fungus *Dematium nigrum*.

The *Aerobacter* obviously provides the forage for the myxamoebae and the fungus produces something, or in some way alters the environment in a manner which permits the slime mold to complete the fructifying process. We do not comprehend the phenomenon, but we are working on the problem.

Sorocarp formation in *Acytostelium leptosomum* is no less interesting. This new and as yet undescribed genus and species was likewise isolated by Miss Fennell and me from forest soil in the conventional way—except that we did not immediately realize the treasure that had been found. As seen in the original source plate, fruiting structures resembled delicate sorocarps of *D. minutum* and it was not until we examined it microscopically that we realized it was something new—a *Dictyostelium* lacking the "dictyo." Unlike any known member of the group, *its sorophores contain no cells* and are inconceivably delicate, consisting of thin rods or tubes measuring from 1 to 2 μ (rarely more) in diameter and devoid of apparent taper except for a barely perceptible thinning in the area of the sori and a limited expansion immediately adjacent to the point of attachment to the substratum. The spores are globose as in *D. lacteum* and vary considerably in size, ranging mostly from 5.0 to 8.0 μ.

Obviously *Acytostelium* presents some unique problems in sorocarp formation. Since it produces a non-cellular stalk, certain of the basic hypotheses justifiably propounded by Miss Fennell and me (**1952**) for *D. discoideum*, and with modifications applied to other species of *Dictyostelium* and *Polysphondylium*, are hardly applicable to this genus. Certainly, vacuolating stalk cells cannot provide the primary lifting force during culmination, for there are no cells in the stalk to expand. Actually we know very little about the building process in the new genus. At first glance one might suppose that here the sorogen must of necessity

ascend the lengthening sorophore as a consequence of the coordinated and cumulative amoeboid movements of its individual cells. Possibly it does. However, fixed and stained whole mounts of developing structures strongly suggest that the great bulk, if not all, of the constituent cells are oriented transversely, or in the same manner as the cells in

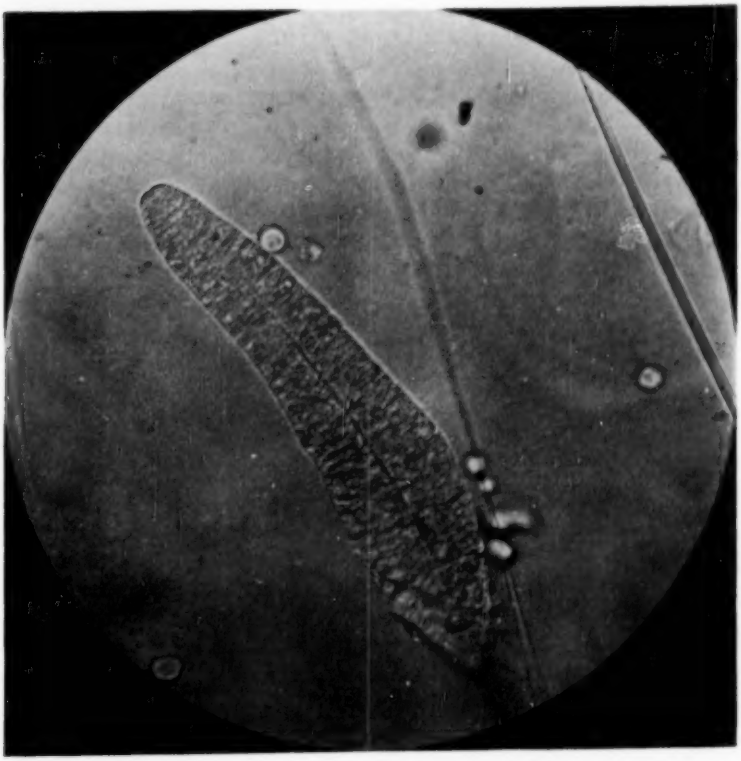

FIG. 6. Developing sorocarp of *Acytostelium leptosomum*. Note the noncellular character of the sorophore at extreme right and the transverse orientation of the myxamoebae in the rising sorogen (center). ×650.

the apical tip of a *D. discoideum* sorocarp, hence in a direction opposite to that which would be anticipated if they were being propelled upward by active individual amoeboid movements (FIG. 6). Possibly the sorophore in *Acytostelium* is really comparable to the sorophore sheath in other species, but with this difference: In *Acytostelium* it is produced

in the center of the mass but is devoid of a lumen, or possesses an infinitely narrow one, so that it presents the appearance of a delicate solid bar. Stained with chloroiodide of zinc and other appropriate stains it gives a cellulose reaction. It dissolves readily in Schweitzer's reagent and 72% H_2SO_4. It is birefringent under polarized light, but being of slight diameter the birefringence appears weak. It thus possesses the basic chemical characteristics of the sorophore sheath in forms previously studied. How does it originate? We do not know, but possibly its formation is catalyzed by and is deposited adjacent to the innermost walls of critically placed and transversely oriented myxamoebae in essentially the same manner as described for *D. discoideum* by Raper and Fennell (**1952**) and more recently amplified by Bonner *et al.* (see p. 189). What provides the necessary force to lift the sorogen if this does not arise through the amoeboid movements of individual cells? Again we do not know, but possibly the cohesive properties of the rising sorogen tend to crowd it upward, and as this occurs the delicate stalk may be continually extended near the apical end. But enough of speculation. We are now attempting to resolve these and other questions and I hope that we will in time find the correct answers to them.

Sufficient has been said to indicate that *Acytostelium* is a genus apart from the cellular slime molds previously known. Yet it is unmistakably a *bona fide* member of the Acrasieae. The myxamoebae feed, grow, and aggregate in a manner common to all. Somewhat suggestive of *D. lacteum*, the sorocarps are typically gregarious in that a dozen or more often arise near the convergence of the inflowing streams of a single radiate pseudoplasmodium (FIG. 4, C). The sorophores appear comparatively long considering their diameter, for they commonly attain a length of 1.5 to 1.8 mm. They are never branched and are terminated by small globose sori commonly measuring 40 to 60 μ. They are extremely ephemeral, collapsing immediately when the cover of the culture dish is removed, thus rendering their study a painstaking and time-consuming task.

The discovery of *Acytostelium* and the limited insight which we now have regarding the fructifying process in this genus would not seem in any way to contradict or obviate what has previously been postulated for *D. discoideum*. It seems rather to demonstrate forcefully that Nature was not limited to any single pattern of morphogenesis when these simple metaphytes were being evolved. Van Tieghem's *Acrasis* (**1880**) and *Coenonia* (**1884**) would undoubtedly afford two additional patterns of developmental behavior (see Raper, **1940**). If only we could rediscover them!

Since the topic of our symposium is physico-chemical control of differentiation, and I freely admit that I have spent much of my time delving in pure speculation, possibly I should return to two additional concrete examples of such effects. The first of these has to do with pigmentation of the spores in *Dictyostelium purpureum*. In a chance observation a few months ago it was noticed that some of the sori were white in a culture plate of this species to which the stain rose bengal, as used in soil microbiology, was added. This preparation consists of 1% dye and 5% phenol in an aqueous solution. It was soon determined that the phenol was responsible for the lack of pigmentation and, additionally, that the effect could not arise as the result of increased acidic conditions in the culture medium. Absence of spore color resulted from the presence of the volatile compound. In one and the same culture exposed only to phenol vapor, normal sorocarps with white sori would develop at a certain distance from the added phenol depending upon its concentration, while outward from this zone the sori became increasingly colored until at the edge of the culture dish they were deep purple and wholly characteristic of the species. It should be recorded that spores from white sori yield typically pigmented cultures when transplanted to suitable cultures in the absence of added phenol. Thus it was established that we were not witnessing a permanent genetic mutation, but rather a situation wherein the presence of the phenol apparently interfered with some enzymatic process essential to pigment formation. Doctor Whittingham of my laboratory has recently examined the matter more carefully, and has tested a considerable number of related compounds to determine, if possible, the chemical bases for the inhibition. His unpublished results may be summarized as follows:

"It appears that a hydroxyl group of the benzene ring is necessary for this phenomenon. Furthermore, substitution at the ortho positions of phenol destroys the desired activity of the compound, whereas substitution at the meta positions does not affect the activity. Apparently, substitution of a non-polar group at the para position of phenol does not affect the activity, as evidenced by the results obtained with p-cresol. However, substitution of a polar group at the para position, as in p-nitrophenol or hydroquinone, drastically changes the effects. Since the minimum concentrations of p-cresol, and resorcinol which duplicate the 'phenol effect' compare favorably with that of phenol, it is probably that all of these compounds are acting in the same manner."

The second example relates to the formation of so-called macrocysts (Raper, **1951**) in certain strains of *Dictyostelium mucoroides*. These structures, apparently observed by Brefeld (**1869**) and termed "dwarfed

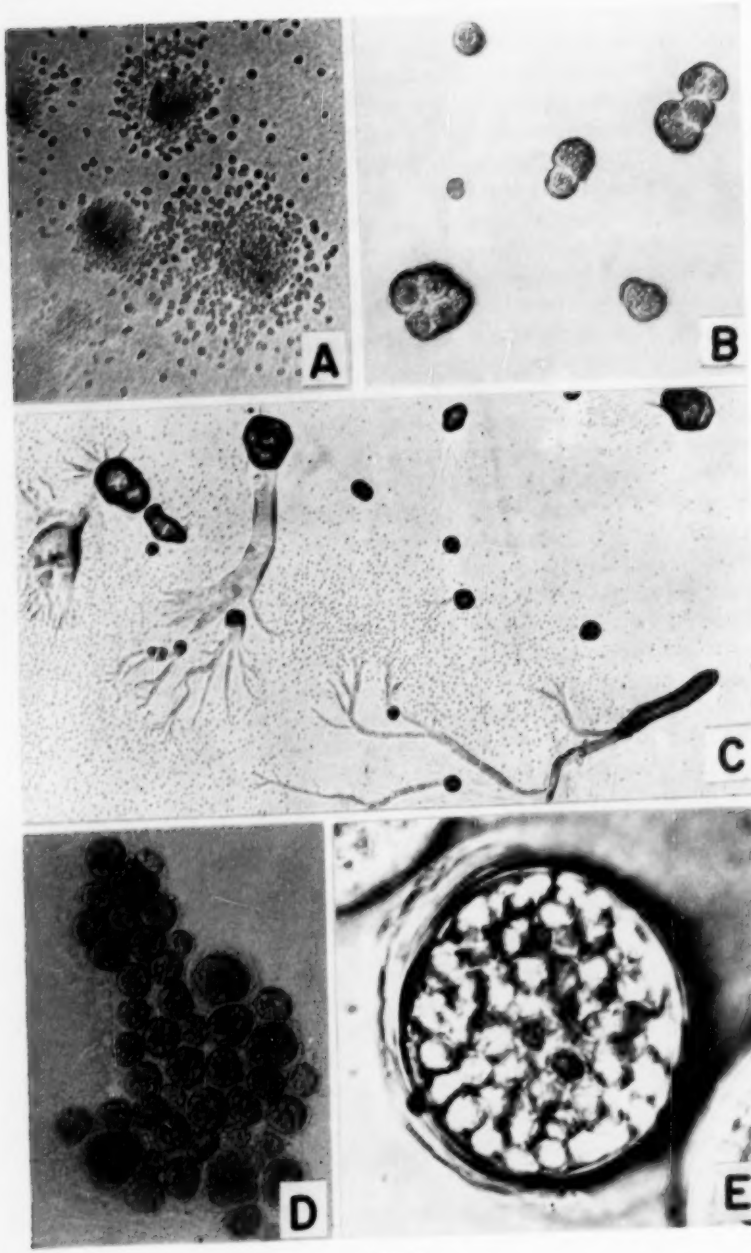

FIG. 7.

sporangia" by him, seemingly represent an aberration of the normal fructifying process which occurs in occasional cultures of *D. mucoroides* and *D. minutum*. They are not developed normally in the vast majority of isolates of either species, which I presume accounts for the fact that they were not reported by Olive (**1902**), Potts (**1902**), Arndt (**1937**), or again by any other student of this group until we began to seek and study large numbers of strains several years ago. Conditions governing macrocyst formation are not completely understood and seem to vary with different strains. Miss Cormier, of my laboratory, has worked particularly with two strains of *D. mucuroides*, S-*28b* and WS-*47*, and I shall discuss these briefly. The macrocysts may be described as limited groups of myxamoebae which, following aggregation (FIG. 7, A), become compacted together and, instead of proceeding to sorocarp formation in the normal way, surround themselves with a comparatively thick and somewhat fibrous envelope which reacts as cellulose. The cells within this wall are at first amoeboid and difficultly discernible, the whole content presenting rather the appearance of a homogenous and finely granular protoplasmic mass. As maturation progresses, somewhat thickened and refractive walls appear to develop around centrally placed cells (FIG. 7, B) and subsequently the entire content of the macrocyst becomes thus differentiated, at this stage appearing to consist of a substantial number (hundreds) of closely compacted and somewhat angular cells (FIGS. 7, D and E). With only limited success thus far we have attempted to explore their possible role in the life cycle of the strains which produce them.

But to return now to the circumstances which favor the production of macrocysts. In strain S-*28b* macrocyst formation is strongly influenced by the temperature of incubation. When the stock culture is grown in the laboratory at 22–23° C some normal sorocarps are produced, but an equal or greater number of myxamoebae contribute to the formation of abundant macrocysts. At 25° C many more macrocysts and virtually no sorocarps are produced, although total growth of the slime mold remains approximately the same. At 15° C, on the other

FIG. 7. Macrocyst formation in *Dictyostelium mucoroides*: A, B, and E, strain S-*28b*; C and D, strain WS-*47*. A. Small aggregating pseudoplasmodia preparatory to macrocyst formation, × 125. B. Macrocysts in process of maturation, × 125. C. A single field in which macrocysts (upper left and right) and sorocarps are developing from superficially similar aggregating pseudoplasmodia, × 25. D. A cluster or packet of mature macrocysts filled with firm-walled cells, or endocytes, × 170. E. Detail of a mature macrocyst—note the heavy cellulosic wall which envelops the macrocyst, × 850.

hand, very few or no macrocysts develop, whereas well-formed sorocarps are produced abundantly; here again total growth is believed to reach approximately the same level, but proceeds at a much slower rate. By dilution plating of spores Miss Cormier (**1955**) has succeeded in isolating clonal sub-strains with markedly different characteristics. For example, a so-called "cyst-former" has been isolated which produces nothing but macrocysts at the temperature of the laboratory. The same strain at 15° C produces mostly sorocarps but also macrocysts in much greater numbers than the original stock. Conversely, another strain, termed a "spore-former," produces sorocarps only at 15° C; at 20° C abundant sorocarps and relatively few macrocysts are produced; whereas at 25° C very few sorocarps and abundant macrocysts are formed. Thus we see in this culture a measure of genetical control, while superimposed on this is a very marked response to a physical factor, namely temperature. The responses in strain WS-*47* are evident but much less marked. The two strains differ in other regards. In S-*28b* the aggregates leading to macrocyst formation are generally quite small and often give rise to single structures, and seldom to groups exceeding eight or ten in number. In contrast, strain WS-*47* commonly develops small but well formed radiate pseudoplasmodia which apparently give rise either to a sorocarp of normal pattern or a cluster of macrocysts varying in number up to twenty-five or more (FIGS. 7, C and D). We have no idea at present concerning the subtle differences which exist between two neighboring and outwardly similar aggregates, one of which may produce a well-formed fruiting structure, the other a platelet of macrocysts (FIG. 7, D). We would like to know.

I have touched upon different facets of the developmental history of the simple slime molds. I have perhaps answered a few questions; I have probably raised many more. This has been done deliberately, for I sincerely believe that the cellular slime molds have much to teach us, if we could but comprehend what we are able to observe. Phenomena of growth and intercellular organization leading to structural differentiation are here presented in a relatively simple and essentially dissected form. We should be able to understand them. Yet, the Acrasieae are living organisms, and the growth and behavior of living things are never really simple, their complexity being more and more evident as we study them with increasing care. This, however, should not discourage us. The cellular slime molds are still simple when compared with any other organisms which attain a multicellular organization. If we continue to pose questions and to seek answers to them through

appropriate experimentation, we may yet obtain some insight into basic vital phenomena of much wider application. It is a goal worth seeking.

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STUDIES ON THE DEVELOPMENT OF ASEXUAL REPRODUCTIVE STRUCTURES IN PILOBOLUS [1]

ROBERT M. PAGE [2]

(WITH 13 FIGURES)

Pilobolus, a fungus assigned to the Phycomycete order Mucorales, is characterized by its black, heavily cutinized sporangia which are forcibly discharged from the sporangiophore at maturity. Under natural conditions, *Pilobolus* grows on dung, and the discharged sporangia may strike and adhere firmly to leaves. If a leaf with adherent sporangia is eaten by a grazing animal, the spores pass unharmed through its digestive tract and emerge in the dung where they germinate to produce mycelia. Under suitable conditions, the mycelium forms sporangia which complete this asexual reproductive cycle.

The formation and discharge of sporangia marks the culmination, not only of the asexual reproductive cycle, but also of a developmental sequence. The first step in this sequence is the formation of swollen sporangial primordia known as trophocysts. Trophocyst formation is followed by the initiation and elongation of sporangiophores and finally by the differentiation of the sporangium and sub-sporangial swelling.

The asexual reproductive structures of *Pilobolus* offered several advantages for a study of development. First, each of the stages is distinctive and easily recognized. Second, there were indications from studies on the periodic formation of sporangia (McVickar, **1942**; Klein, **1948**; Schmidle, **1951**) and from a nutritional study (Page, **1952**) that the developmental sequence might be shifted or interrupted by changing environmental factors. Lastly, as a result of nutritional studies (Page, **1952**; Hesseltine *et al.*, **1953**; Neilands, **1953**), it is now possible to grow *Pilobolus* on a medium of known chemical composition and observe

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the effect of alteration of chemical as well as physical factors in the environment.

The objective of the present study was to gain insight into the mechanism of the development of the early stages of the asexual reproductive apparatus by manipulation of the physical and chemical factors of the environment. While these experiments have not been carried far, the results are presented in the hope that they will give further indication of the potentialities of fungi as experimental material for investigation of the refractory problems of morphogenesis.

MATERIALS AND METHODS

The strain of *Pilobolus* employed for this study was the same one whose nutritional characteristics have been reported (Page, **1952**). This fungus, which was tentatively identified as *P. kleinii* van Tieg., was isolated from horse dung in 1949. It has been maintained in the laboratory on dung extract agar and on synthetic media by repeated transfer. Although this strain was originally selected for its stability in culture as well as for its rapid and vigorous growth, it has recently shown a tendency to produce sectors of moribund mycelium. The frequency of these sectors appears to be reduced by occasional inoculation of stock cultures with spores rather than with hyphal tips; however, most stock cultures and all experimental cultures were inoculated with hyphal tips contained in small blocks of agar cut from the periphery of mycelia grown in the dark.

The basal media for all experiments contained (per liter): L-asparagine, 7.55 g; sodium acetate ($CH_3COONa \cdot 3H_2O$), 10 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; micronutrients (Ryan *et al.*, **1943**); and either hemin (10 mg) or ferrochrome (0.1 mg). The substitution of ferrochrome for hemin did not affect the rate of mycelial growth in the dark, but the inhibition of growth by light previously reported (Page, **1952**) did not occur when hemin was replaced by ferrochrome. Unless otherwise noted, the basal media also contained thiamine—either as the hydrochloride or as the mononitrate—in a concentration of 10 mg per liter.

For experiments, *Pilobolus* was grown either in growth tubes or in modified culture tubes. The use of growth tubes permitted measurement of the rate of growth as well as observation of the reproductive structures. The use of modified culture tubes facilitated counting the number of trophocysts, and had other advantages which will be pointed out in later sections. These tubes were prepared by heating 15 × 125 mm Pyrex cultures tubes and indenting one side of each tube to form

a dam approximately 5 mm high, 55 mm from the bottom of the tube. Six-tenths of a milliliter of medium was placed in each of these tubes, which were kept horizontal during incubation.

After inoculation, cultures were grown in the dark at about 25° C. After 3–4 days (growth tubes) or 4–5 days (culture tubes), appropriate cultures were exposed to light with an intensity of approximately 150 foot-candles supplied by fluorescent tubes. The cultures were then returned to the dark, and the number of trophocysts in each culture was routinely counted 48 hours later.

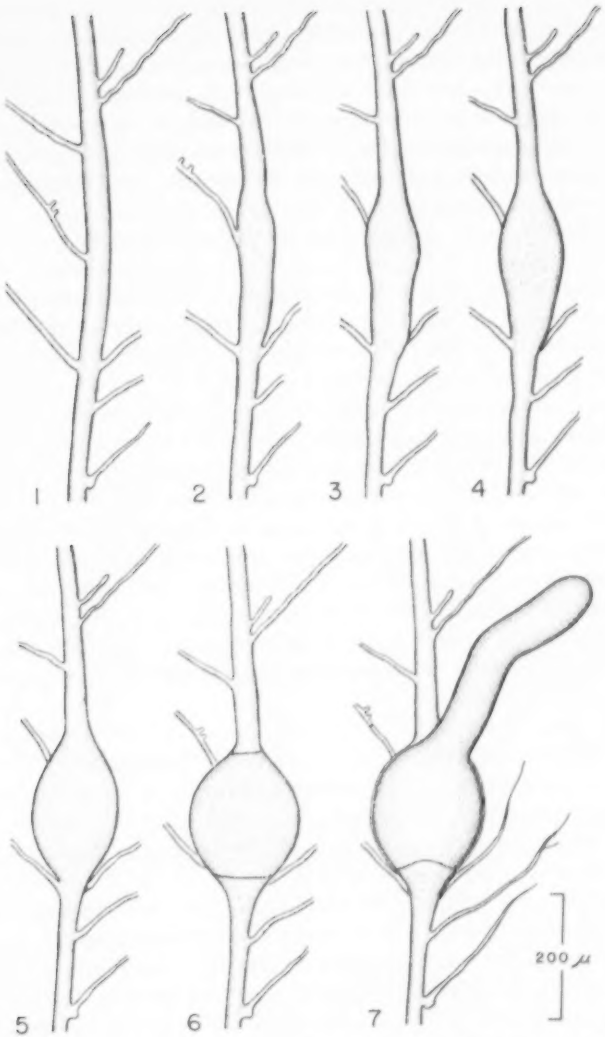
TROPHOCYST FORMATION

Morphology of trophocyst formation. The development of trophocysts in the strain of *Pilobolus* used for this study was followed in cultures which had been allowed to grow in the dark on synthetic medium for five days and were then exposed to diffuse daylight in the laboratory. Periodic observation of the cultures showed that although the time required for trophocysts to reach certain stages varied widely, the general course of events was very similar to that shown in FIGS. 1–7 and to that described by McVickar (**1942**).

Two observable processes are involved in the formation of a trophocyst. The first—which might be considered a type of morphogenetic movement—is a flow of protoplasm toward the site of trophocyst development. The second process is the synthesis of new wall material.

The first indication of trophocyst formation is the aggregation of dense, granular, yellow protoplasm at the point in a hypha where a trophocyst is destined to form (FIG. 1). What factors determine where trophocysts will form are not known, but if, as Lyr (**1954**) has pointed out, trophocysts tend to be terminal or intercalary according to the species, it is possible that their location is in part determined by heredity.

Within a few hours, the hypha becomes distended as further yellow, granular protoplasm flows into the developing trophocyst (FIGS. 2–5). It is interesting to note that the hypha does not appear to swell evenly, but rather to bulge in places, and then these swellings coalesce as the trophocyst continues to enlarge. The increase in the area of the hyphal wall to accommodate the increasing volume of protoplasm does not appear to be accomplished by a simple stretching or a smooth process of intussusception, but rather by a localized rupturing coupled with a synthesis of new wall material, because the wall of an enlarging trophocyst often appears scaly during a period of rapid growth. The wall of a mature trophocyst is considerably thicker than the wall of a hypha and appears to be laminated.



FIGS. 1–7. Successive stages in the development of a trophocyst in *Pilobolus kleinii*. Drawings were made with the aid of a camera lucida 6, 7, 8, 9, 14, 32, and 51 hours respectively after illumination of a culture grown on synthetic medium for five days in the dark, × 95.

Sooner or later cross-walls are laid down which delimit the trophocyst from the rest of the hypha (FIG. 6). In this case, the cross-walls were not completed until 32 hours after the culture had been illuminated, but in other cases they have appeared to be complete after 10 hours. The completion of cross-walls marks the end of trophocyst formation, but later the sporangiophore is initiated, and a prominent central vacuole which extends from the trophocyst out into the elongating sporangiophore becomes visible (FIG. 7).

Action spectrum for trophocyst formation. In cases in which light affects a process it is frequently possible to gain some insight into the mechanism of the process by determining the action spectrum. Since light was required for the formation of trophocysts by this strain of *Pilobolus* (Page, **1952**), it was of interest to determine the action spectrum for trophocyst formation.

For determination of the action spectrum, the monochromator from a Beckman model DU spectrophotometer was used as a source of monochromatic light. The output of this monochromator at various wavelengths was measured in arbitrary units by means of a Photovolt vacuum tube photometer over part of the range of the monochromator, and the calibration curve thus obtained was conservatively extrapolated on a basis of the emission spectrum of a tungsten-filament lamp to cover a range of 390–700 mμ. Exposures were calculated to give equal amounts of radiant energy at each of the wavelengths used. At each wavelength, the slit width of the monochromator was adjusted to give an effective band width of 10 mμ.

The cultures used for the determination of the action spectrum were grown in the dark for five days in modified culture tubes. When each tube was raised to a vertical position to insert it in the monochromator, the mycelium adhered to the wall of the tube, while the medium drained to the bottom of the tube where it did not intercept the beam of light from the monochromator, so that errors caused by fluorescence or absorption by the medium were eliminated. The cultures were returned to the dark for 48 hours before trophocysts were counted.

The results of one trial are shown in FIG. 8. While the variation in the number of trophocysts formed in replicate cultures was too great to permit any significance to be attached to the precise shape of the curve or to the positions and relative heights of the various peaks, it was apparent from this and other trials that light of wavelengths longer than 510 mμ was not effective in inducing this strain of *Pilobolus* to form trophocysts. It was not possible to delimit accurately the shortest wavelength which would stimulate the fungus to produce trophocysts, because

absorption of light by the Pyrex glass culture tubes becomes considerable in the ultra-violet. It is clear from the data shown in FIG. 8 and that obtained in other trials that light with wavelengths between 380–510 mμ was effective in inducing the formation of trophocysts.

The fact that trophocysts were induced by light of wavelengths between 380–510 mμ suggested that either a carotinoid or a flavin might be the substance which absorbs the light to initiate trophocyst formation. β-carotene, for example, absorbs light strongly in this range (Karrer, **1948**), and β-carotene has been demonstrated in *Pilobolus* (Bünning,

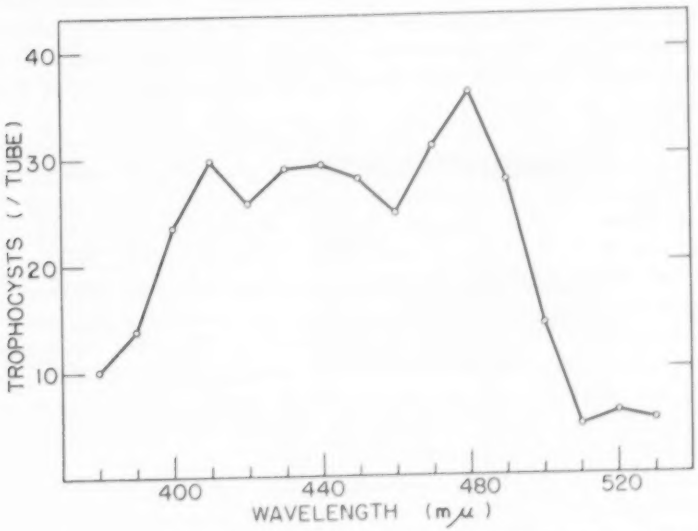


FIG. 8. Action spectrum for trophocyst formation in *Pilobolus*. Each point represents the mean number of trophocysts formed by four replicate cultures.

1937). Riboflavin also absorbs light strongly in this portion of the spectrum and has been shown to play a role in the phototropism of higher plants (Galston and Baker, **1949**). Since the action spectra obtained were not sufficiently critical to provide any indication which—if either—of these classes of compounds might be involved, attempts were made to distinguish between the two by adding to the medium (a) substances reported to stimulate synthesis of one or the other class of compounds or (b) substances which would inhibit the synthesis or action of flavins or carotinoids.

Stimulatory compounds. Various substances which have been re-

ported to modify or enhance the synthesis of carotinoids were added to the basal medium in an attempt either to induce the formation of trophocysts in the dark or to increase the number of trophocysts formed by cultures after brief exposure to the light. Commercial carotene (plain, dissolved in olive oil, or suspended with Tween 40 or Polyethylene Glycol 300), β-ionone, actidione, leucine, and various concentrations of thiamine were tried without success. None of the compounds induced mycelia to form trophocysts in the dark, and only high concentrations of thiamine increased the number of trophocysts formed by cultures exposed briefly to light. This increase produced by thiamine was not great, nor was it accompanied by any visible change in the color or intensity of pigmentation. The results of these trials, therefore, provided no evidence for distinguishing between carotinoid and flavin systems.

Inhibitors. The use of selective inhibitors appeared to offer considerable promise as a means for determining whether the light required for the induction of trophocysts in this strain of *Pilobolus* was absorbed by a flavin or by a carotinoid, because effective inhibitors have been reported for both classes of compounds. Goodwin (**1952**) has demonstrated that diphenylamine in a concentration of 1–40,000 almost completely repressed the formation of α-, β-, and γ-carotene and lycopene in *Phycomyces blakesleeanus*, a member of the Mucorales that appears to be quite closely related to *Pilobolus*. Snell *et al.* (**1953**) found that high concentrations of L-lyxoflavin (10–50 μg/ml) inhibited the growth of *Lactobacillus casei*. From the fact that this inhibition could be reversed by the addition of riboflavin (0.8–1.0 μg/ml) to the medium, Snell *et al.* concluded that lyxoflavin acted as a competitive inhibitor for riboflavin in *L. casei*. The effect of lyxoflavin appears to vary in different organisms, because Snell *et al.* found that it served as a growth factor for *L. lactis*, and other workers have reported that growth of rats, baby pigs, and poultry was stimulated by the addition of lyxoflavin to relatively crude diets (cf. Cheldelin and King, **1954**).

Preliminary tests. Before diphenylamine and lyxoflavin could be used as tools for investigating the mechanism of trophocyst formation, it was necessary to determine their effects on growth and pigmentation. It was particularly important to obtain this information in the case of lyxoflavin, because of the differences in its effects which have been reported for different organisms.

The results of one of these preliminary trials indicated that lyxoflavin strongly inhibited the growth of *Pilobolus* when added to the synthetic basal medium in a concentration of 100 μg/ml, but that the growth rate

in the presence of 1.0 or 10 μg/ml was not significantly different from that on the unsupplemented basal medium. Further, lyxoflavin inhibited growth much more strongly than did isoriboflavin in an equal concentration.

The results of a second trial carried out with growth tubes in which the synthetic medium was supplemented with L-lyxoflavin (100 μg/ml), riboflavin (10 μg/ml), lyxoflavin plus riboflavin, and, for purposes of comparison, diphenylamine (25 μg/ml) are shown in FIG. 9. The results of this trial confirmed the inhibition of growth by lyxoflavin and demonstrated that this inhibition could be reversed almost completely by riboflavin. Diphenylamine also inhibited growth to some extent, but the most striking effect of this compound was on pigmentation. The cultures were exposed to light for 10 seconds 7 days after inocula-

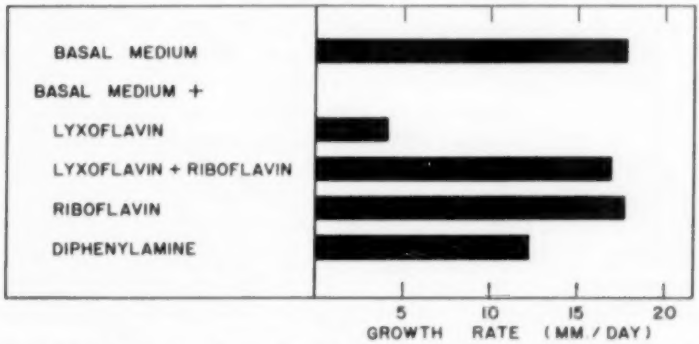


FIG. 9. Effect of lyxoflavin, riboflavin, and diphenylamine on the rate of growth of *Pilobolus*. The length of each bar represents the mean growth rate of four replicate cultures.

tion, and 48 hours later, the hyphae and trophocysts formed on the basal medium and those containing lyxoflavin and riboflavin were bright yellow. By way of contrast, the hyphae, trophocysts, and later the sporangia, which were formed in the medium with diphenylamine, were completely colorless.

It was clear from these results that this strain of *Pilobolus* resembled *Lactobacillus casei* in that high concentrations of lyxoflavin inhibited growth, in that this inhibition could be reversed by riboflavin, and in that low concentrations of lyxoflavin did not stimulate growth in the absence of added riboflavin. It was also evident that—as far as could be determined visually—the synthesis of carotinoids was strongly inhibited by diphenylamine.

It was also apparent from the results of preliminary trials that the effect on trophocyst formation of compounds which inhibited growth was difficult to evaluate when the fungus was grown in growth tubes, because the mycelia were of different sizes at the time they were exposed to light and at the time trophocysts were counted. This difficulty was overcome by the use of the modified culture tubes. The small volume (0.6 ml) of medium in each tube acted as a limiting factor, so that even

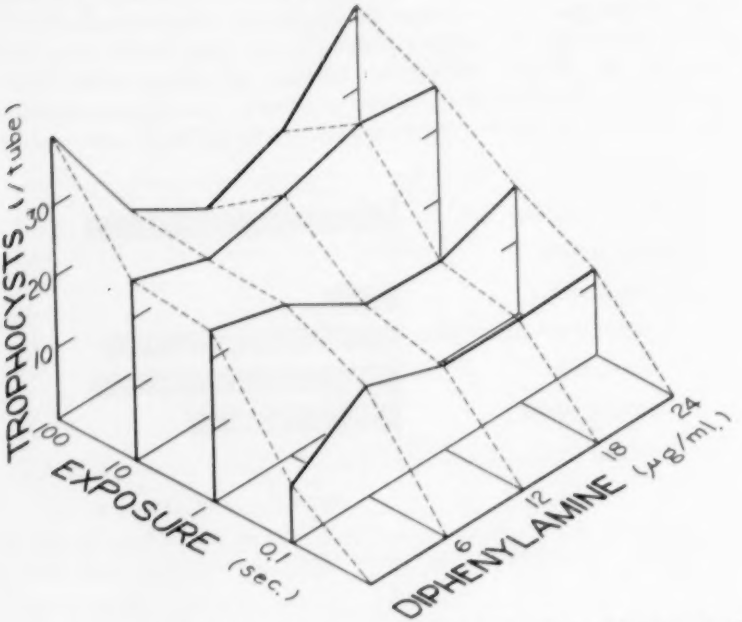


FIG. 10. Effect of diphenylamine concentration and duration of exposure of mycelium to light on trophocyst formation in *Pilobolus*. Each point represents the mean number of trophocysts formed in four replicate cultures. Isometric projection.

mycelia which grew at very different rates reached very nearly the same size by the time they were exposed to light.

Effect of diphenylamine on trophocyst formation. The effect of diphenylamine on trophocyst formation was tested by adding four concentrations of diphenylamine (6, 12, 18, and 24 μg/ml) to the basal synthetic medium. After they had grown for five days in the dark, replicate cultures with the basal medium and each concentration of diphenylamine were exposed to light for approximately 0.1, 1, 10, and

100 seconds. The cultures were returned to the dark for 48 hours after which the number of trophocysts in each tube was counted.

The results of one trial with diphenylamine are shown in FIG. 10. No trophocysts were formed by cultures which were not exposed to light. In cultures which were exposed to light, the number of trophocysts at each concentration of diphenylamine was increased by increased duration of exposure to light. At most of the exposures, increasing the concentration of diphenylamine had little effect on the number of trophocysts formed. The apparent inhibition of trophocyst formation by some of the concentrations of diphenylamine in cultures exposed for 100 seconds would seem to be an exception, but it is probable that this dip in the curves is the result of variability. In all of the experiments in which diphenylamine was added to the medium, variability among replicates was very high. It is notable that when the results of three trials similar to this one were averaged and plotted, a prominent hump occupied approximately the same position as the depression shown in FIG. 10.

While it was clear from the results of this and other trials that diphenylamine had no consistent effect on trophocyst formation, it was equally clear that diphenylamine inhibited carotinoid synthesis to a marked degree. Trophocysts formed in media containing 6 μg of diphenylamine per milliliter were a considerably paler yellow than those formed by mycelia grown in the basal medium, and those formed in media with 12 μg/ml were virtually colorless. No trace of yellow pigment was visible at the two higher concentrations of diphenylamine; both hyphae and trophocysts appeared to be completely colorless. Diphenylamine, then, inhibited carotinoid synthesis but had no clear effect on trophocyst formation other than to increase the variability between replicates.

Effect of lyxoflavin on trophocyst formation. The effect of lyxoflavin on trophocyst formation was tested by the same technique used with diphenylamine. L-lyxoflavin (Merck) was added to the synthetic medium to give concentrations of 25, 50, 75, and 100 μg/ml. Replicate cultures with each of these media and the basal medium were exposed to light for approximately 0.1, 1, 10, and 100 seconds. Parenthetically, it will be noted that in this experiment a few trophocysts were formed in cultures which were not exposed—or at least not deliberately exposed—to light. Although these and other cultures were inoculated and handled only in red light from a photographic safelight, it is probable that some of the cultures in this and the next experiment were exposed to light from a Bunsen burner at the time of inoculation.

As shown in FIG. 11, the results of this experiment indicated clearly

that lyxoflavin inhibited trophocyst formation. At each duration of exposure, the number of trophocysts formed was decreased by an increase in the concentration of lyxoflavin. With short exposures, trophocysts were suppressed by high concentrations of lyxoflavin, while with longer exposures, the number formed was greatly reduced. It is also evident from FIG. 11 that at each concentration of lyxoflavin, increase in the duration of exposure results in an increase in the number of trophocysts formed; therefore, increased exposure to light is capable of overcoming to some extent the inhibition by lyxoflavin.

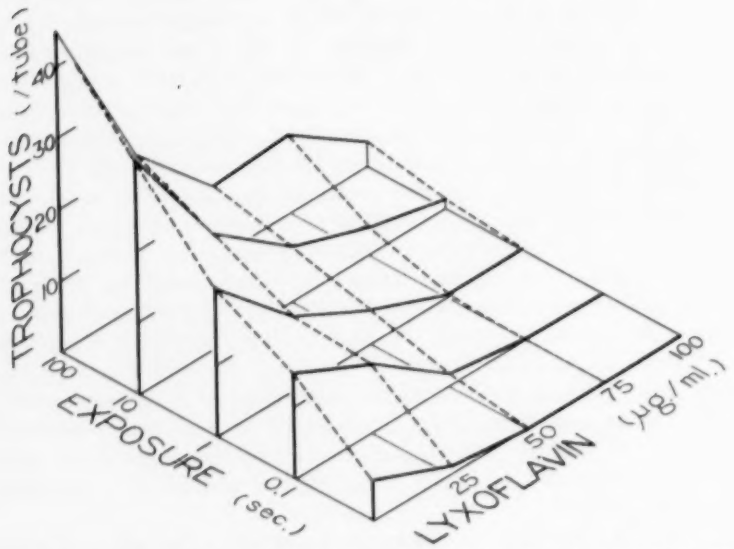


FIG. 11. Effect of lyxoflavin concentration and duration of exposure of mycelium to light on trophocyst formation in *Pilobolus*. Each point represents the mean number of trophocysts formed in four replicate cultures. Isometric projection.

The fact that the inhibition of trophocyst formation by high concentrations of lyxoflavin could be overcome to some extent by increasing the duration of exposure of the mycelium to light is significant for two reasons. First, it shows that strongly inhibited mycelia are neither dead nor are they incapable of forming trophocysts under any circumstances. Second, the modification of the effect of lyxoflavin by changes in the length of exposure is evidence that lyxoflavin must interfere with the substance that actually absorbs the light to initiate trophocyst formation. If the substance with which lyxoflavin interferes were involved in some

later stage of trophocyst development, changing the duration of exposure should have had no effect on the number of trophocysts formed.

Since lyxoflavin inhibited trophocyst formation, it was of interest to determine whether this inhibition could be relieved by riboflavin. Accordingly, media were prepared which contained lyxoflavin (100 μg/ml), riboflavin (10 μg/ml), and a combination of the two. The cultures were allowed to grow in the dark for four days and were then exposed in replicate to light for approximately 0.1, 1, 10, and 100 sec. Trophocysts were counted 48 hours after exposure.

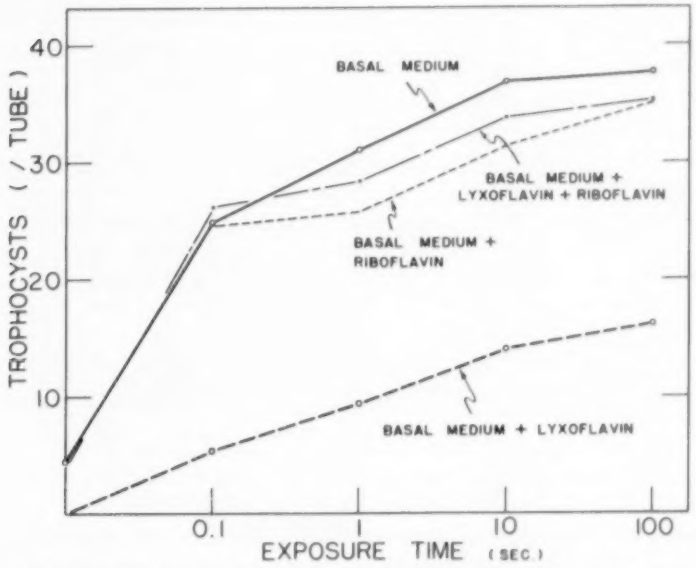


FIG. 12. Effect on trophocyst formation of lyxoflavin (100 μg/ml), riboflavin (10 μg/ml), and lyxoflavin plus riboflavin on trophocyst formation by mycelia exposed to light for various lengths of time. Each point represents the mean number of trophocysts of five replicate cultures.

The results (FIG. 12) demonstrated that the inhibition of trophocyst formation by lyxoflavin was relieved by the simultaneous addition of riboflavin to the medium. Lyxoflavin alone greatly reduced the number of trophocysts formed; however, the number of trophocysts formed at each time of exposure by mycelia growing in media with both lyxoflavin and riboflavin was nearly the same as the number formed on the basal synthetic medium.

The fact that the number of trophocysts formed in media containing lyxoflavin plus riboflavin was not greatly different from the number formed on the basal medium is of additional significance, because it showed that lyxoflavin was not merely acting as a colored filter to reduce the amount of light of effective wavelengths received by the mycelium. Media containing lyxoflavin plus riboflavin contained a higher concentration of light-absorbing compounds than those with lyxoflavin alone, yet trophocyst formation was not inhibited.

The results of this experiment also indicated that addition of riboflavin to the medium did not increase the sensitivity of the mycelium to light. The numbers of trophocysts formed in media containing riboflavin were, in fact, slightly smaller than those formed in the basal medium. These results might be interpreted as suggesting that riboflavin is not the flavin which absorbs light to initiate trophocyst formation, but there are at least two alternative explanations. One possible explanation for the failure of added riboflavin to increase the numbers of trophocysts formed would be to assume that the concentration of riboflavin in the hyphae is optimal, so that additional riboflavin has no effect. Another possible explanation would be to assume that the riboflavin that is functional in the absorption of light is combined—possibly as a phosphate, as a nucleotide, or with a protein—and that the other component of the combination is limiting. In this connection it might be mentioned that in one trial, the addition of adenine, adenosine, or adenylic acid to media containing riboflavin did not increase the number of trophocysts formed.

Discussion. While the results of this investigation do not prove that absorption of light by a flavin initiates the formation of trophocysts in this strain of *Pilobolus,* all of the evidence obtained is compatible with this view. The action spectrum for trophocyst formation agrees with the absorption spectrum of flavins and carotinoids. Lyxoflavin which has been reported to inhibit riboflavin by competition (Snell *et al.,* **1953**) inhibits growth of *Pilobolus* as well as trophocyst formation; whereas, diphenylamine, a carotinoid inhibitor, had no consistent effect on trophocyst formation. Furthermore, the inhibition of both trophocyst formation and growth by lyxoflavin is almost completely reversed by riboflavin. The action spectrum and the effects of inhibitors suggest strongly that it is a flavin—possibly riboflavin—within the hyphae that absorbs light to initiate trophocyst formation in this strain of *Pilobolus.*

Either of two roles might be assigned to the flavin. When hyphae are exposed to light, the flavin might synthesize an inducing substance

or it might destroy an inhibiting substance. All attempts to demonstrate a substance capable of inducing the formation of trophocysts in the dark have been unsuccessful. Cultures in liquid media have been grown in the light for various lengths of time and under various conditions. The medium and extracts made in various ways from mycelia from these cultures were added to cultures grown in the dark, but in no case were trophocysts formed.

It is possible that the reason for the failure to demonstrate a trophocyst-inducing substance is that it is labile. The fact that when a portion of a mycelium was exposed to light, trophocysts were formed only on the portion of the mycelium that was exposed suggests that any trophocyst-inducing substance might either be labile or else that it might not be translocated.

The best precedent for the participation of a flavin in a photochemical reaction is the photoinactivation of auxin by riboflavin which Galston and Baker (**1949**) have proposed as the mechanism for phototropism in higher plants. If a similar mechanism were involved in trophocyst formation in *Pilobolus,* it would be necessary to assume that auxin inhibits trophocyst formation; moreover, it would be resonable to predict that anti-auxins might stimulate trophocyst formation. Neither auxin (indole-3-acetic acid) nor anti-auxins (2, 4 dichloroanisole; 2, 4, 6 trichlorophenoxyacetic acid; p-chlorophenoxyisobutyric acid) in various concentrations induced the formation of trophocysts in the dark, nor did they have any marked effect on the numbers formed by cultures after they had been exposed to light.

SPORANGIOPHORE INITIATION AND GROWTH

The stages in the development of the asexual reproductive apparatus which immediately follow trophocyst formation in *Pilobolus* are (a) the initiation of sporangiophores, and (b) the elongation of the sporangiophores. While observations on these stages have been only preliminary, they indicate that these stages are susceptible to analysis by the alteration of physical and chemical factors in the environment.

In an earlier study (Page, **1952**) it was found that two factors—thiazole and light—were involved in the development of sporangia from trophocysts but it was not determined at what stage each of these factors became operative.

Effect of thiazole. If cultures of *Pilobolus kleinii* were grown on a basal medium lacking thiamine in a daily alternation of 8 hours of light and 16 hours of darkness, no sporangia were formed; whereas, in

media containing thiamine, abundant sporangia were formed under the same conditions. Further trials disclosed that it was the thiazole moiety of the thiamine molecule which was required for sporangium formation.

In later observations, many short sporangiophores could be seen in cultures grown in media lacking thiazole in a daily alternation of light and darkness. Thiazole, therefore, would appear to be involved not in the initiation of sporangiophores, but rather in their elongation. The fact that sporangiophores require an external source of thiazole for growth whereas vegetative hyphae do not, indicates that the reproductive apparatus has an obvious biochemical differentiation which accompanies morphological differentiation.

Effect of light. When cultures of this strain of *Pilobolus kleinii* were grown in continuous light, the mycelia formed many trophocysts but few sporangia (Page, **1952**); therefore, light appeared to inhibit sporangium formation. When these cultures grown in continuous light were examined, very few sporangiophores were observed, so that the stage that is inhibited by continuous light would appear to be the initiation of sporangiophores. This inhibition of sporangiophore initiation was seen even more strikingly in two strains (probably *P. crystallinus* (Fries)) from Alaska which produced no sporangiophores whatsoever under continuous illumination.

If, one the other hand, cultures of *P. kleinii* were exposed to light only briefly and were then returned to the dark, many trophocysts but few sporangia were formed. For example, in one trial with growth tubes, cultures were exposed to light on the fourth day after inoculation for times which ranged from 15 seconds to 256 minutes. The average number of trophocysts formed in each of four replicate tubes ranged from 41 to 69, while the average number of sporangia counted five days after exposure ranged from 0.25 to 1.8.

From the fact that few sporangiophores were initiated in continuous light or in darkness, it was concluded that both darkness and light were required for the initiation of sporangiophores. These relationships were clarified by experiments in which cultures of *Pilobolus* were subjected to two exposures to light.

In one experiment, the effect on sporangium formation of changing the length of a dark period between two exposures was tested. Cultures grown in the dark in growth tubes were exposed to light for two minutes, were returned to the dark for times which varied by two-hour intervals from 2 to 18 hours, and were again exposed to light for two minutes. Trophocyst counts made 24 hours after the first exposure showed that approximately the same number of trophocysts was formed in all cases.

By 24 hours after the second exposure, an average of three sporangia was formed by each culture in which 16 hours separated the exposures and 12 in each culture with an 18-hour interval. No sporangia were present in any of the other cultures.

In a second experiment with two exposures, the effect of changing the duration of the second exposure was tested. Cultures grown in the dark for four days were exposed to light for two minutes and returned

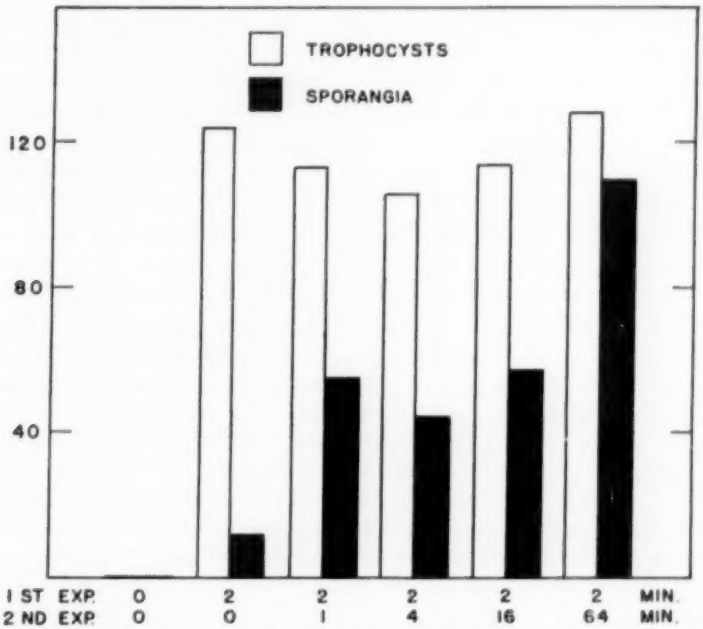


FIG. 13. Effect on sporangium formation of changing the duration of the second of two exposures to light separated by 19 hours of darkness. Counts of trophocysts were made 18 hours after the first exposure, and counts of sporangia were made 48 hours after the first exposure. The height of each bar represents the mean of four replicate cultures grown in growth tubes.

to the dark for 19 hours. The cultures were then exposed to light a second time for 1–64 minutes. The results of trophocyst counts made 18 hours after the first exposure and counts of sporangia made 48 hours after the first exposure are shown in FIG. 13. The fact that the number of sporangia formed was proportional to the duration of the second exposure indicates that exposure of trophocysts to light stimulates the formation of sporangia.

From the trials with two exposures and the observations on the behavior of cultures grown in continuous light or in continuous darkness, it may be concluded that light stimulates the initiation of sporangiophores from trophocysts, but that the illumination of trophocysts must be preceded by a period of darkness. From the fact that a 16–18-hour dark period separating exposures increased the number of sporangia, it would appear that the trophocysts required this length of time to attain physiological maturity, although they appeared to be morphologically mature in 10 hours in this case. Possibly some substance must accumulate in the trophocysts before they become sensitive to light. Since few or no sporangiophores were initiated by trophocysts subjected to continuous illumination, this substance must either not be formed in light or be light-labile. This requirement for a dark period preceding illumination is quite the reverse of the requirement of *Choanephora cucurbitarum* (Barnett and Lilly, **1950**) in which conidiophores are formed when a light period precedes a dark period.

CONCLUSIONS

The following tentative outline for the early stages of the development of the asexual reproductive apparatus in the strain of *Pilobolus* studied might be formulated on a basis of the results of this investigation. When hyphae of the vegetative mycelium are exposed to light, a flavin in the hyphae absorbs light and induces the formation of trophocysts. Trophocysts accumulate in the dark a substance of unknown nature which sensitizes them to light. If sensitized trophocysts are exposed to light, sporangiophores are initiated. After their initiation, sporangiophores require an external source of thiazole for their elongation.

Further experimentation will be required to test the validity of these suggestions and to extend the analysis to the later stages of sporangium development. Although the conclusions drawn from the results obtained thus far are highly tentative, the results affirm that *Pilobolus* is excellent material for the study of problems of development.

SUMMARY

The formation of trophocysts and sporangiophores in a strain of *Pilobolus kleinii* which formed trophocysts only after the mycelium had been exposed to light was investigated by altering physical and chemical factors in the environment. A determination of the action spectrum showed that light with wavelengths between 380 and 410 mμ was effective in inducing the formation of trophocysts. Since carotinoids and

flavins absorb light in this portion of the visible spectrum, an effort was made to determine which of these classes of compounds was involved by the use of inhibitors. Diphenylamine, which inhibits the formation of certain carotinoids, had no consistent effect on trophocyst formation although pigment formation was suppressed. L-lyxoflavin, which has been reported as a competitive inhibitor of riboflavin, strongly inhibited trophocyst formation. This inhibition of trophocyst formation by lyxoflavin was overcome in part by increasing the duration of exposure of the mycelium to light and was almost completely reversed by addition of riboflavin. The results of trials with inhibitors suggested that it is a flavin rather than a carotinoid in the mycelium of *Pilobolus* which absorbs light to initiate trophocyst formation. Investigation of the factors affecting sporangiophore initiation by trophocysts showed that (a) few sporangia were produced by trophocysts allowed to develop either in darkness or in continuous light, and (b) when cultures were exposed to light twice, the number of sporangia was proportional to the duration of the second exposure. These results were taken to indicate that light stimulates the initiation of sporangiophores by trophocysts and that the exposure of trophocysts to light is most effective if preceded by a period of darkness.

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THE RELATION BETWEEN CELLULAR METABOLISM AND MORPHOGENESIS IN BLASTOCLADIELLA [1, 2]

EDWARD C. CANTINO

(WITH 4 FIGURES)

The process of growth and development exhibited in the life history of every living individual is an orderly and highly oriented mechanism. In higher plants and animals, a single, fertilized egg cell gives rise to the many organs and tissues characteristic of the adult individual as the striking phenomenon of differentiation gradually unfolds and culminates in the formation of the arms and the legs, the lungs and the heart, in man—or the stems and the roots, the sepals and the petals, in flowering plants.

The same basic phenomenon of growth and differentiation is also displayed by the most primitive, microscopic plants and animals. But in many such creatures, organismal architecture is at a much lower level of complexity; it is therefore more readily amenable to experimental investigations under the kind of controlled, environmental conditions which cannot be readily attained with most higher organisms. The aquatic mold *Blastocladiella emersonii*, which occurs in stagnant and slow-moving bodies of fresh water, is such an organism.

That genesis of form occurs is an indisputable fact, but the riddle of "why" and "how" it occurs—perhaps the greatest puzzle to confront biology today—remains almost a complete mystery. It seems reasonable to expect that, at one level of integration at least, the study of metabolism should provide us with some of the answers. Indeed, microbial physiology has been studied most extensively and the abundance of precise, definitive information derived therefrom has revealed many intricate details of the numerous pathways of cellular metabolism. And yet, in contrast to this admirable and stimulating state of affairs, very little is

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known about the causal role of intracellular biochemical processes in the fabrication of cellular and organismal architecture. From certain points of view, *B. emersonii* is ideally suited for studying problems of this sort. It was for this reason that, in 1949, we initiated our investigations of the relation between metabolism and morphogenesis in *Blastocladiella;* we welcome this opportunity to attempt to present a brief but, hopefully, an integrated view of what we have learned since that time.

This small (ca. 150 to 200 μ), non-filamentous fungus, whose life history (FIG. 1) begins with a uniflagellate, swimming cell, can develop into either of two quite different kinds of plants, each of which consists of only two cells—an upper, spherical sac or sporangium, and a lower, highly-branched, root-like stalk or rhizoidal system. In one plant type, the terminal sac is a deep brown resistant sporangium which possesses a thick, heavily-pitted wall impregnated with melanin; the protoplasm enclosed therein contains small quantities of γ-carotene. In the other plant type, the terminal sac is a distinctly-different kind of sporangium which displays conspicuous discharge papillae and possesses a thin, unpitted, colorless wall devoid of melanin; the protoplast within it is apparently devoid of γ-carotene (Cantino and Horenstein, **1955, 1956**).

Attempts to establish whether or not this ontogenetic pattern was under the influence of external, environmental conditions led to the discovery (Cantino, **1951**) that the mode of development could, indeed, be controlled at will. In the presence of approximately 10^{-2} M sodium bicarbonate on agar media, the uniflagellate, motile cells always settled down, retracted their flagella, and developed into the brown, thick-walled, resistant-sporangial plants; in the absence of added bicarbonate on otherwise identical (cf. Addendum to paper) media, the thin-walled, colorless varieties were formed instead. Subsequent studies of a second species of *Blastocladiella,* similar in many essential respects to *B. emersonii,* revealed that it also responded to bicarbonate in precisely the same way (Cantino and Hyatt, **1953a**).

Several questions of a rather broad scope naturally arose immediately. For example: (1) The resistant sporangium seemed to contain larger quantities of discrete, fat-like globules than did the thin-walled sporangium. (2) The wall of the resistant sporangium, which apparently consisted essentially of chitin, was much thicker than that of the thin-walled sporangium. (3) Both visual observations and chemical analyses (Cantino and Horenstein, **1955, 1956**) revealed that the wall of the resistant sporangium was impregnated with a melanin-like pigment and that the protoplast within it contained γ-carotene, whereas the thin-walled sporangium possessed neither of these constituents. Thus,

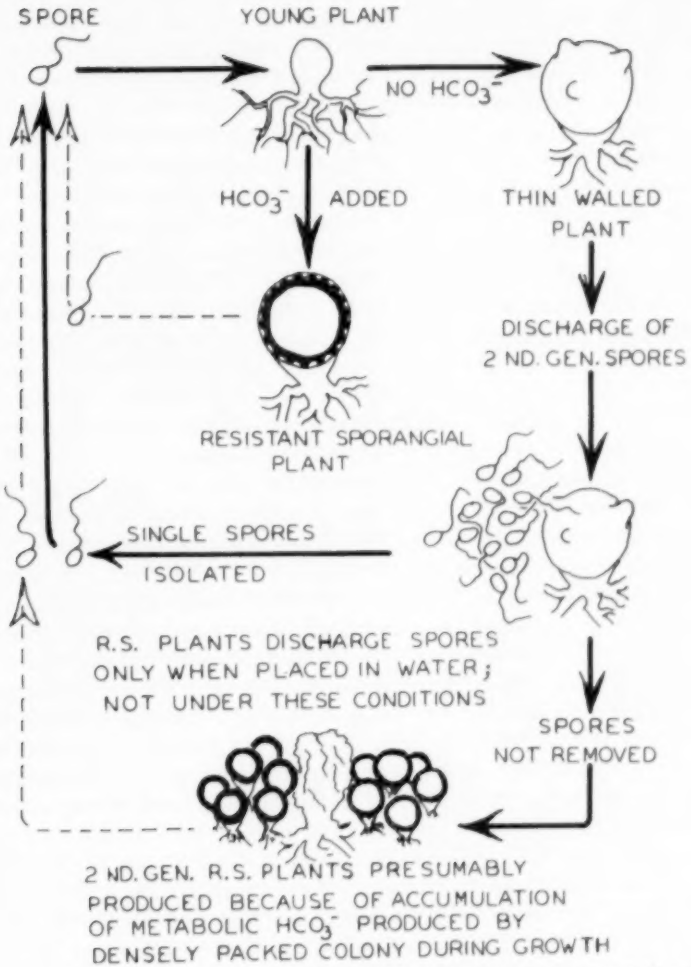
SPORE
YOUNG PLANT
NO HCO_3^-
HCO_3^- ADDED
THIN WALLED PLANT
DISCHARGE OF 2 ND. GEN. SPORES
RESISTANT SPORANGIAL PLANT
SINGLE SPORES ISOLATED
R.S. PLANTS DISCHARGE SPORES ONLY WHEN PLACED IN WATER; NOT UNDER THESE CONDITIONS
SPORES NOT REMOVED
2 ND. GEN. R.S. PLANTS PRESUMABLY PRODUCED BECAUSE OF ACCUMULATION OF METABOLIC HCO_3^- PRODUCED BY DENSELY PACKED COLONY DURING GROWTH

FIG. 1. The two, alternate, developmental pathways in *Blastocladiella emersonii* leading to the formation of (1) thin-walled, papillate plants, and (2) thick-walled, brown, pitted, resistant sporangial plants.

the morphogenetic mechanism leading to the formation of a resistant sporangium appeared to be associated with the production of increased quantities of lipoidal and chitinous materials, and it was clearly associated with a *de novo* synthesis of γ-carotene and melanin.

We therefore asked ourselves: first, were all of these changes an intrinsic part of, and indispensable for, morphogenesis; and second, if they were not, was it possible to demonstrate that one or more of these biosynthetic mechanisms could be inhibited without seriously interfering with the overall developmental pattern responsible for the structure and function of the final product, the resistant sporangium? It was then learned that at least one of these reaction chains—that leading to the synthesis of melanin—could be uncoupled *in vivo* from the rest of the metabolic machine by incorporating phenylthiourea (ca. 2×10^{-3} M) in the medium during growth (Cantino, **1953**). Under these conditions, bicarbonate continued to induce the formation of structurally-normal and perfectly-viable, but absolutely colorless, resistant sporangia which were visibly devoid of melanin. These experiments led us to suspect that it would be possible to further dissect and elucidate *in vivo* the mechanism by which the metabolic machinery of *B. emersonii* initiates and maintains the genesis of a resistant sporangium.

A second rather general question of this sort also posed itself: for the formation of resistant sporangia, was the external bicarbonate required throughout ontogeny or was it needed only for a limited and specific period of time during development? It was then established that bicarbonate was indispensable only for the first three-fifths of the generation time; beyond this critical period, the morphogenetic mechanism became irreversible and the newborn, developing terminal cell matured into a resistant sporangium whether or not bicarbonate was then removed from the immediate environment. Judging from the indirect evidence available (Cantino, **1952**), it was concluded that the phenomenon was directly related to a progressive decrease in the permeability of the thallus to bicarbonate during the early differentiation and subsequent delimitation of the resistant sporangium. In other words, this barrier imposed a gradually-increasing restriction upon the quantity of external bicarbonate that could penetrate the thallus membranes from the outside, and concomitantly a restriction upon the quantity of metabolic bicarbonate produced internally which could escape to the outside. Thus, it followed that the biosynthetic reactions leading to the synthesis of chitin, fat, melanin, carotene, and in fact the resistant sporangium itself—reactions induced and maintained by increased concentrations of bicarbonate—were assured continuity by a kind of "intracellular autocatalysis" until differentiation was complete.

Finally, having completed these exploratory experiments and having already established *in vivo* a quantitative relationship between the external stimulus and the morphogenetic response, attempts were made to

elucidate and to unravel that aspect of the problem which appeared to us to be of greatest importance. That is, what was the biological significance of the bicarbonate "trigger mechanism" in terms of the more fundamental, biochemical reactions that it motivated?

At the very start, the indirect evidence derived from our studies of the effects of tricarboxylic acid cycle intermediates and metabolic inhibitors on growth had led us to believe that the focal point for the bicarbonate effect was the ketoglutarate oxidase system (Cantino, **1951**; cf. also Cantino, **1953**). We concluded, as a working hypothesis consistent with data then available, that increased concentrations of bicarbonate within the organism tended to slow up, stop, or perhaps even reverse the oxidative decarboxylations mediated by isocitric dehydrogenase and ketoglutarate oxidase. (It was suggested that the activity of the ketoglutarate oxidase might have been biotin-dependent.) It followed, then, that bicarbonate would have caused some piling up of intermediates in the tricarboxylic acid cycle between ketoglutarate and its C6, or C2 and C4, precursors in the cycle. Therefore, we also suggested that the accumulation of these Krebs cycle intermediates somehow initiated the various shunt mechanisms leading to the biosynthesis of melanin and the other ingredients obviously associated with the genesis of a resistant sporangium.

Subsequently, some of these notions were put to a direct test. For example, the thin-walled, papillate, colorless plants of *B. emersonii* were incubated in the presence of bicarbonate for short time intervals and then quickly killed and homogenized. Standard chromatographic and spectrophometric analyses then revealed (Cantino, unpublished) that the concentration of total keto-acids found therein was at least twice as great as that found in homogenates derived from *Blastocladiella* which had been incubated in the absence of bicarbonate under otherwise identical conditions. Most of the increase was due to the accumulation of α-ketoglutaric acid. Thus, these data provided us with direct evidence for some of the conclusions theretofore based upon results (Cantino, **1951**) of *in vivo* studies with proliferating plants. Subsequent, more definitive experiments (Cantino and Horenstein, **1956**) with radioactive CO_2, non-proliferating plant suspensions, and cell-free extracts have corroborated these observations.

At this point, two spontaneous mutants of *Blastocladiella* were discovered and isolated from the wild type (Cantino and Hyatt, **1953a**). These mutant varieties synthesized copious quantities of an orange pigment which consisted almost exclusively of γ-carotene. Thus, they were easily distinguished at a glance from the parental *Blastocladiella*. But

of utmost significance and utility was the fact that the mutant strains could not respond to the bicarbonate stimulus in that they did not produce the brown, resistant sporangia in the presence of bicarbonate (cf. FIG. 1). In contrast to the wild types, their mode of development was limited to but a single morphogenetic pathway which led only to the formation of thin-walled, papillate plants. Assuming that our line of reasoning was correct, therefore, two conclusions were drawn from these observations: (1) that in these mutant organisms, the presumed locus for the bicarbonate "trigger mechanism"—ketoglutarate oxidase—was either missing or inoperative; and (2) that synthesis of γ-carotene, which occurred both in resistant sporangia and in the mutant plants, might have been due to a block in the tricarboxylic acid cycle, but it was not due to the specific, additional reactions (*e.g.* CO_2 fixation; see later) directly induced by the bicarbonate itself. The fortuitous appearance of these mutants provided us with alternative methods by which we set out to establish whether or not our notions about the nature of the bicarbonate "trigger mechanism" were correct.

To begin with, it followed that it should be possible—indeed, that it would be necessary—to demonstrate *in vitro:* (1) that the wild type *Blastocladiella* possessed the enzymatic activities usually associated with the enzymes of the tricarboxylic acid cycle and, in particular, that ketoglutarate could be reductively carboxylated to isocitrate; and (2) that in the mutant strains of *Blastocladiella* which could not respond to bicarbonate the presumed locus of the bicarbonate effect—ketoglutarate oxidase—was indeed absent or inoperative. *In vitro* studies with cell-free preparations were therefore initiated. The more definitive, direct evidence derived therefrom is summarized below; it has amplified and significantly strengthened our concepts regarding the role of bicarbonate in the development of *Blastocladiella.*

Spectrophotometric, manometric, and direct chemical assays (Cantino and Hyatt, **1953b**; Brown and Cantino, **1955**) with cell-free preparations of the wild type revealed the presence therein of the enzymatic, oxidative activities usually associated with cytochrome, oxidase, aconitase, TPN-specific isocitric dehydrogenase, TPN- (and/or DPN-) dependent ketoglutaric acid oxidase, succinic dehydrogenase, fumarase, and a DPN-dependent malic dehydrogenase (cf. FIG. 2 and TABLE I). Furthermore, as postulated during our *in vivo* studies (Cantino, **1951**), biotin appeared to play some kind of a stimulatory role, though possibly an indirect one, in the *in vitro* oxidation of ketoglutarate. On the other hand, similar assays with cell-free preparations of the mutant strain of *Blastocladiella* revealed the presence therein of all of these same enzy-

matic activities except aconitase and ketoglutarate oxidase. Thus, when considered as a functional unit, the two successive oxidative decarboxylations in the sequence of reactions in the tricarboxylic acid cycle leading from cis-aconitate to succinate were operative in the wild type but inoperative in the mutant. Thus, the comparative study of the wild type *Blastocladiella* and the mutant variety derived therefrom had pro-

TABLE I

A summary of the oxidative activity of cell-free preparations (from Cantino and Hyatt, **1953b**) of *Blastocladiella emersonii* and the mutant strain derived therefrom. The representative data were selected from numerous experiments among which the results never deviated by more than about 10 percent from the values reported here. Units of activities are all corrected for endogenous levels obtained in the absence of substrate.

Units of cytochrome oxidase activity represent changes in optical density ($\times 10^3$) at 550 mμ brought about by 0.3 ml of supernatant in a cuvette containing 4.4×10^{-2} M phosphate and 2.8×10^{-5} M reduced cytochrome C in a total volume of 3.0 ml. Reaction time: 10 minutes.

Units of succinoxidase, isocitric dehydrogenase, ketoglutarate oxidase, malic dehydrogenase, and fumarase activities represent changes in optical density ($\times 10^3$) at 550 mμ brought about by 0.3 ml of supernatant in a cuvette containing 4.4×10^{-2} M phosphate, 2.8×10^{-5} M cytochrome C, 10^{-4} M KCN, and 1.7×10^{-2} M substrate in a total volume of 3.0 ml. In addition, 2.3×10^{-4} M TPN was present for the isocitrate run, and 2.1×10^{-4} M DPN for the malate, fumarate, and ketoglutarate runs. The oxidation of ketoglutarate was measured in the presence of 10^{-1} M malonate. Reaction time for malate and fumarate: 10 minutes. Reaction time for the rest: 20 minutes.

Units of aconitase activity represent changes in optical density ($\times 10^3$) at 340 mμ brought about by 0.6 ml of supernatant in a cuvette containing 4.4×10^{-2} M phosphate, 2.3×10^{-4} M TPN, and 1.7×10^{-2} M cis-aconitate in a total volume of 3.0 ml. Reaction time: 25 minutes.

Substrate	Corrected activity	
	B. emersonii	BEM
reduced cytochrome C	111	114
succinate	125	126
isocitrate	160	156
malate	220	74
fumarate	148	70
cis-aconitate	108	0
ketoglutarate	94	less than 4

vided strong, corroborative evidence for our concept of the biochemical basis for morphogenesis in *B. emersonii*.

Next, attempts were made to substantiate our belief that the piling up of ketoglutarate in the organism was coupled in some manner to the induction of new biosynthetic pathways; *e.g.* those leading to the synthesis of melanin during morphogenesis. According to our hypothesis, it seemed likely that the enzyme complex responsible for melanin synthesis in resistant-sporangial plants would be absent or at least inopera-

tive in the thin-walled, colorless plants of *Blastocladiella* which had not been subjected to the bicarbonate "trigger mechanism." Analyses then showed that this assumption was correct. Using manometric, spectrophotometric, and chronometric techniques and several methods of pre-

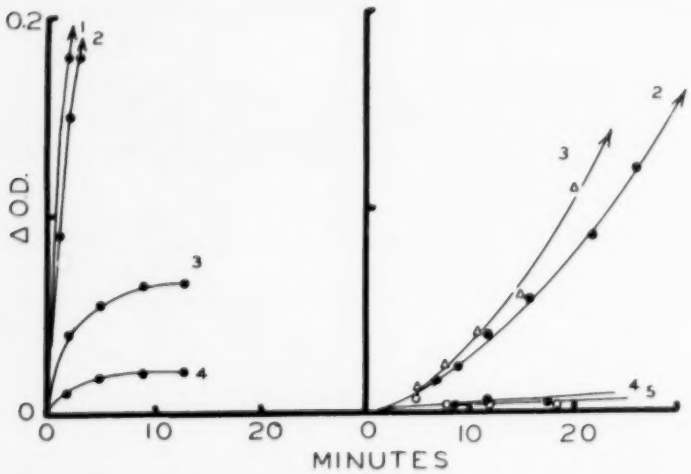

FIG. 2. Representative results illustrating the oxidation of tricarboxylic acid cycle intermediates by cell-free preparations (according to Cantino and Hyatt, 1953b) of thin-walled plants of *B. emersonii*. *Left:* oxidation of malate as measured by the coupled reduction of cytochrome C, *via* carrier quantities of DPN, at 550 mμ. Cuvettes contained 0.3 ml supernatant, 4.4×10^{-2} M phosphate pH 7.0, 2.8×10^{-5} M cytochrome C, 2.1×10^{-4} M DPN, and 10^{-4} M KCN in a total volume of 3.0 ml with exceptions and additions as indicated below. Curve 1: 1.7×10^{-2} M malate, with double strength supernatant. Curve 2: 1.7×10^{-2} M malate. Curve 3: 1.7×10^{-2} M malate but no DPN added. Curve 4: endogenous (no substrate added). *Right:* oxidation of isocitrate and cis-aconitate as measured by the reduction of TPN at 340 mμ. Cuvettes contained 0.3 ml of the supernatant preparation for isocitrate and 0.6 ml for cis-aconitate, 4.4×10^{-2} M phosphate pH 7.0, and 2.3×10^{-4} M TPN, in a total volume of 3.0 ml with exceptions and additions as indicated below. Curve 2: 1.7×10^{-2} M isocitrate. Curve 3: 1.7×10^{-2} M cis-aconitate. Curves 4 and 5: endogenous (no substrate).

paring homogenates, polyphenol oxidase (tyrosinase) activity was never detected in homogenates of thin-walled plants derived from cultures of various ages grown under different environmental conditions (Cantino and Horenstein, 1955). However, as pointed out above, these same cell-free extracts displayed vigorous cytochrome oxidase activity and

they were capable of oxidizing all of the presumed intermediates of the tricarboxylic acid cycle.

But, following imposition of the bicarbonate "trigger mechanism" upon young thin-walled plants, the cytochrome oxidase, aconitase, succinic dehydrogenase, ketoglutarate oxidase, fumarase, and malic dehydrogenase activities either disappeared or were rendered quite impotent; at least, they were now no longer detectable with our assay procedures. On the other hand, the enzyme which would be required to initiate reversal of the tricarboxylic acid cycle if bicarbonate were involved in a reductive carboxylation of ketoglutarate—*i.e.* isocitric dehydrogenase—remained fully functional. At the same time, a polyphenol oxidase (tyrosinase) did make its appearance, and it was invariably found in abundance in the mature, brown, resistant sporangia which developed in response to the presence of bicarbonate. Most of the polyphenol oxidase was localized in, and apparently firmly bound to, the wall of the resistant sporangium, but part of it could be recovered in dialyzed, supernatant fractions free of cell-wall fragments. Like other polyphenol oxidases, it was completely inhibited by copper reagents such as phenylthiourea. However, although the enzyme complex has not yet been purified extensively, it appears to be rather atypical in its behavior; it not only mediated electron transfer between substrate and oxygen, but also between substrate and TPN (but not DPN).

Finally, it was clearly established (FIG. 3) that ketoglutarate was required for optimum activity of this polyphenol oxidase system, whether it was assayed manometrically *via* oxygen consumption or spectrophotometrically *via* reduction of TPN or formation of colored quinones. Ketoglutarate appeared to function as an essential factor for polyphenol oxidase activity by virtue of its concomitant reduction *via* the TPN-specific isocitric dehydrogenase. Preliminary analyses had not established clearly the fate of ketoglutarate during the oxidation of polyphenol substrates (Cantino and Horenstein, **1955**). It now seems probable, however, that added ketoglutarate was being metabolized and, simultaneously, partially regenerated by way of an S. K. I. (succinate-ketoglutarate-isocitrate) cycle (cf. Cantino and Horenstein, **1956**, for details).

If it is assumed that this "polyphenol oxidase" complex, whose characteristics were studied *in vitro*, was also the agent responsible for melanin synthesis *in vivo*, then these results provided direct evidence for our notions that: (1) initiation and maintenance of melanin synthesis during morphogenesis was dependent upon, and coupled directly with, the availability of accumulated ketoglutarate; and (2) this, in turn, was

induced by the presence of increased concentrations of bicarbonate which not only brought the tricarboxylic acid cycle to a standstill but, indeed, induced a partial reversal (cf. discussion in Cantino, **1955**, and Cantino and Horenstein, **1955**, and particularly the evidence derived from studies by Cantino and Horenstein, **1956**, with labeled bicarbonate and labeled glucose).

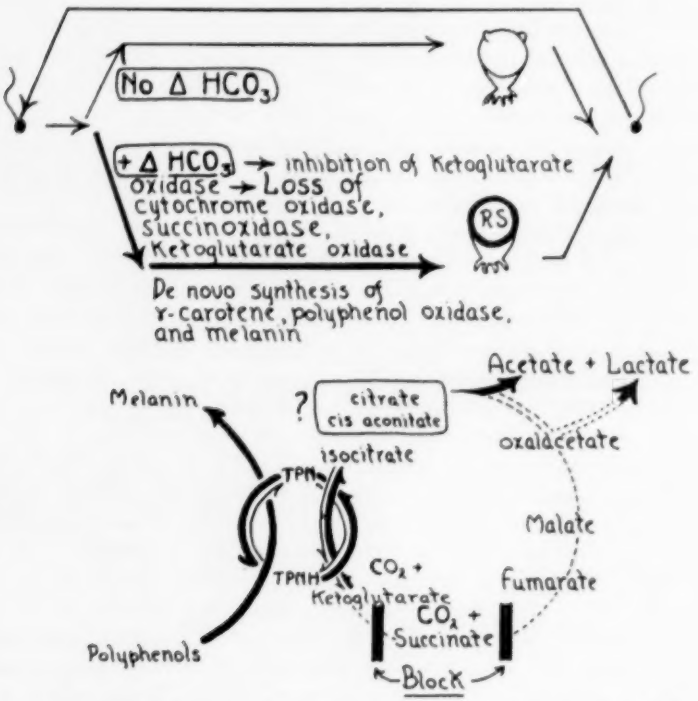

FIG. 3. Schematic representation of the relation between oxidation of polyphenols and the stimulatory action of ketoglutarate thereon.

It seems reasonably certain that the tricarboxylic acid cycle is, at most, a weakly-functional oxidative pathway in *B. emersonii* (cf. discussion in Cantino, **1955**). It cannot be responsible for generating more than a very small portion of the energy required for the work performed by the overall metabolic machine of the cell. Nevertheless, it is almost certainly involved in numerous important, and in some cases perhaps rather subtle, roles which are essential for the integrity and the mainte-

nance of the cellular machine from one generation to the next. In this capacity, the tricarboxylic acid cycle appears to serve as a locus for the initiation and subsequent maintenance of the bicarbonate "trigger mechanism" in morphogenesis. During the genesis of a resistant sporangium the tricarboxylic acid cycle, as a functional unit, is destroyed. It reappears once again, however, at the start of the next generation. Thus, the ontogenetic cycle which leads from spores to resistant sporangial plants and then again to spores has a causal counterpart within the cell—the cyclic disappearance and subsequent reappearance of a weakly-functional but nonetheless essential tricarboxylic acid cycle.

And so, developmental patterns in *Blastocladiella* may be summarized as follows (FIG. 1): The uniflagellate swarmers of *B. emersonii* retract their flagella and germinate to produce small, non-filamentous thalli which display a determinate system of growth. In the presence of added bicarbonate on solid media, such thalli develop directly into deep brown, thick-walled, pitted, resistant sporangial plants; their maturation is followed by cessation of growth. But in the absence of added bicarbonate, these same thalli develop instead into plants with distinctly different terminal, thin-walled, papillate sporangia. Following deliquescence of papillae, the latter discharge thousands of second-generation, uniflagellate swarmers over a small area immediately surrounding the parent plant. There, they germinate to produce a colony of second-generation plants. If individual plants are removed from the colony before they mature and are transferred to fresh media, they will again develop into the thin-walled, papillate variety. This can be done repeatedly to yield generation after generation of thin-walled plants. But if they are left in place, the vast majority of them assume the brown, resistant sporangial form. Under these conditions, the growth of the colony ceases after one or two generations. Cessation of growth appears to be due, at least in part, to the accumulation of metabolic bicarbonate in the densely-packed cluster of plants which induces most members of the colony to produce resistant sporangia. The growth of the colony is therefore self-limited; that is, it is terminated because of the liberation of a metabolic product—CO_2—which initiates differentiation of the members of the colony into non-proliferating plants (Cantino, 1951).

On the other hand, the uniflagellate swarmers of the mutant strains of *B. emersonii* cannot develop into resistant sporangial plants even if bicarbonate has been added to the medium; they produce only the thin-walled variety. Therefore, although these small, non-filamentous thalli are also determinate in their growth habit, the colonies derived there-

from are not. The peripheral spread of the mutant colonies, unlike those of the wild type, does not cease after one or two generations; instead, they continue to liberate motile swarmers and thus they proliferate so long as nutrients are available for new cell generations. The mutant *Blastocladiella* has lost the enzymes—ketoglutarate oxidase and aconitase—which, in the wild type, respond to the accumulation of metabolic bicarbonate and thereby impose a limitation on growth by inducing differentiation; the mutants, therefore, continue to proliferate indefinitely.

ADDENDUM

At the time that this report was presented in Berkeley, California, some of our friends (cf. Emerson, **1956**) questioned the validity of our interpretation of the early experiments (Cantino, **1951**) that had formed the basis upon which the whole superstructure of our case had been

TABLE II

Concentration of sodium bicarbonate in the peptone medium before autoclaving	Average percent resistant sporangial plants in populations of *B. emersonii* grown on five different concentrations of the peptone medium
2.2×10^{-2} M	87
1.7×10^{-2} M	84
1.4×10^{-2} M	37
1.0×10^{-2} M	6
3.3×10^{-3} M	0

built; namely, that it was indeed bicarbonate which induced the morphogenetic pattern leading to the formation of resistant sporangia. They suggested that it might have been a change in pH associated with the addition of bicarbonate, and not the bicarbonate *per se*, which induced morphogenesis. They insisted that our published experiments did not render this alternative explanation out of the question; we, on the contrary, insisted that they did. In order to clarify and, if possible, resolve our differences, we submit the following brief resumé of our point of view in the matter.

I. In the very first experiments (Cantino, **1951**) carried out in Delft, *B. emersonii* was grown on a peptone-agar medium to which different quantities of sodium bicarbonate were added before autoclaving. The concentrations of bicarbonate used, and the percent resistant sporangial plants found among the populations of *B. emersonii* grown on these media, are tabulated in TABLE II. The pH of the medium con-

taining 2.2×10^{-2} M bicarbonate was about 8.8 (not reported in Cantino, **1951**); that of the others was not recorded at the time.

From theoretical considerations alone, the variation in pH induced by a decrease in the bicarbonate concentration from 2.2×10^{-2} M to 1.0×10^{-2} M would not have been expected to exceed *a few tenths* of one pH unit. In the presence of the buffer capacity afforded by the peptone medium, it would have been even less. Thus, the absence of resistant sporangial plants in peptone media containing less than 10^{-2} M bicarbonate was ascribed to the lack of sufficient quantities of the bicarbonate itself, not to the existence of a very slightly lowered pH.

II. When sterile solutions of bicarbonate were added to the peptone media after autoclaving, the results were the same as those recorded in TABLE II (Cantino, **1951**). Thus, the bicarbonate "trigger mechanism" could not have been ascribed to the production of some active substance during sterilization.

TABLE III

Concentration of bicarbonate	pH of medium at time of inoculation	pH of medium after 5 days of growth	Percent R.S. plants in population
5×10^{-2} M	8.8	—	No growth
1×10^{-2} M	8.8	8.6	99
5×10^{-3} M	8.7	8.5	65
4×10^{-3} M	8.6	8.3	10
3×10^{-3} M	8.5	8.3	0
2×10^{-3} M	8.3	8.1	0

III. We reported at the Berkeley meetings that in subsequent experiments resistant sporangial plants were not formed on peptone media adjusted to pH 8.8 with alkali, whereas on media containing bicarbonate at pH 8.8 resistant sporangial plants were formed as usual.

It seemed to us that the results itemized above constituted sufficient evidence for our conclusion that the bicarbonate effect could not have been induced by a change in pH. Our critics did not accept this as sufficient evidence; we in turn did not then, and still do not now, understand the reason why. Nevertheless, in order to provide what appears to us to be more than ample evidence that the effective agent is bicarbonate—not pH—we submit the data in TABLE III. For these experiments we used the basal medium which we have employed for the past five years; it consists of 1.25 g Difco peptone, 1.25 g Difco yeast extract, 3.0 g glucose, and 20.0 g Difco agar per liter of distilled water. The concentration of bicarbonate required for optimum production of

resistant sporangial plants on this medium is slightly lower than that which was needed on the Delft peptone medium (Cantino, **1952**). Different concentrations of bicarbonate were added before autoclaving, the pH of the media was taken before inoculation and again after the 5-day growth period at 20° C, and the percent of resistant sporangial plants on the different media was tabulated. Other experiments completed in

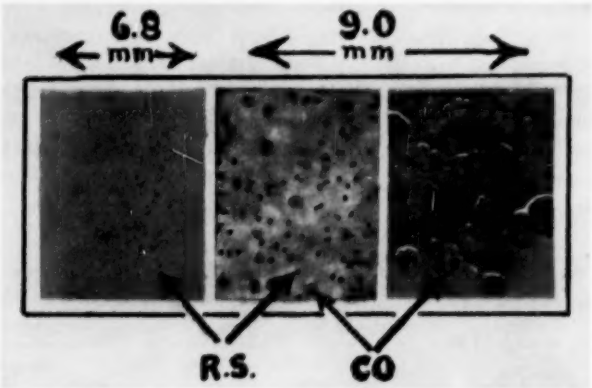

FIG. 4. Populations of *B. emersonii* grown under different environmental conditions on agar media. Left Plate: a 6-day-old population consisting of ca. 100 percent first-generation, individual, brown, thick-walled, resistant-sporangial plants (RS). This is representative of the appearance of populations grown in the presence of 10^{-2} M $NaHCO_3$ (TABLE III). Center Plate: a 6-day-old population consisting of ca. 70 percent first-generation, individual, resistant-sporangial plants and ca. 30 percent colonies (CO) of second-generation, smaller, closely-appressed, resistant-sporangial plants; each colony was derived from spores liberated from a single, first-generation, thin-walled, colorless plant. This is representative of the appearance of populations grown in the presence of 5×10^{-3} M $NaHCO_3$ (TABLE III). Right Plate: a 12-day-old population consisting of ca. 100 percent colonies of second-generation resistant-sporangial plants. This is representative of the appearance of populations grown in the presence of 3×10^{-3} M $NaHCO_3$ (TABLE III).

the past few years have shown that, as was true for the close relative *Blastocladia*, *Blastocladiella* responded to bicarbonate only if the pH of the medium was at a satisfactory level—*e.g.*, in the range between pH 7.8 and 8.8. We submit the data in TABLE III as further evidence that within this range of pH it is bicarbonate and not the reaction of the medium which induces *Blastocladiella* to produce resistant sporangial plants (FIG. 4).

Finally, some of our friends were concerned (cf. Emerson, **1956**) with the apparently anomalous behavior of gaseous CO_2 (Cantino, **1951**). In these experiments, 8 to 10 Petri dishes, each containing about 20 ml of medium, were incubated in 3-liter gas chambers into which the CO_2 was introduced. At concentrations above 5%, CO_2 did not permit growth. At concentrations below 5%, it seldom induced an incidence of resistant sporangial plants above 50%. We do not know for certain why CO_2 was toxic at levels above 5%. However, we think that we are justified in accepting it as a fact without explanation for the moment, insofar as this observation in no way contradicts the results of our investigations with bicarbonate. More important, however, is the fact that it is not difficult to understand why the sub-lethal concentrations of CO_2 below 5 percent did not induce the formation of large quantities of resistant sporangial plants. For example, at a level of 3 percent, the total amount of CO_2 in the chamber was only 4 mM. Even if as much as one-half of this had been absorbed by the 200 ml of medium in the Petri dishes without reducing the pH to undesirable levels, the total dissolved CO_2 would have been no more than 10^{-2} M. It would not have been sufficient to induce the morphogenetic effect (cf. TABLE II).

We trust that the arguments and the additional evidence we have presented will suffice to dispel any notion that a pH effect is responsible for our bicarbonate "trigger mechanism."

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LYSOZYME-INDUCED SENSITIVITY OF NEUROSPORA ASCOSPORES TO FURFURAL [1]

R. J. Lowry, A. S. Sussman and Berta Heidenhain

(with 1 figure)

The precise nature of the permeability barriers of plant cells has eluded analysis although their physiological effects have been exhaustively studied. Contributing most to the difficulties encountered in these studies has been the complexity of their chemical constituents and organization. However, an excellent approach to this problem exists in the use of enzymes as specific reagents in the analysis of cell wall and membrane constituents. Apart from their specificity, these substances act under mild (physiological) conditions and are large enough in size so that it may be assumed that their effect would be localized on the outside of the cell. Weibull (**1953**) has shown that the ability of a bacterial cell to retain certain cellular constituents is greatly altered by the use of lysozyme. In addition, Tomcsik (**1954**) reported that prolongations of bacterial cross walls may extend into the capsule on the basis of enzymatic digestion of antibody-treated cells.

With these facts in mind, studies were undertaken on the effect of enzymatic treatment upon the ascospores of *Neurospora tetrasperma*. These cells exist in at least two different physiological conditions, the durations of which are under the control of the investigator. Thus, in the dormant state, the cell metabolizes at a low rate, is impermeable to some poisons, and is resistant to drying and other physical treatments. Two means exist for breaking dormancy and these include heat-treatment (Goddard, **1935**) and treatment with furfural and related heterocyclic compounds (Emerson, **1948**; Sussman, **1953**). Upon such activation a twenty to thirty-fold increase in metabolic rate ensues, accompanied by other changes in permeability and resistance to chemical and physical treatments. Using these facts as a basis, the effect of lysozyme and other enzymes upon the activation and germination of ascospores of *N. tetrasperma* was investigated. By this means it was hoped to learn

[1] We would like to express our gratitude for the generous grant from the Rackham Fund of the University of Michigan which made this research possible.

something about the chemical nature of the cell wall, and whether a change in it occurred as a result of the activation process.

MATERIALS AND METHODS

Ascospores were obtained in the manner outlined by Goddard (**1935**). Strains *374* and *377* of *N. tetrasperma*, obtained through the courtesy of Dr. B. O. Dodge, were crossed and grown at 25° C. The ascospores were stored at 4° C in closed bottles, under which conditions only a slow decline in the ability to germinate resulted. Heat-activation was accomplished by suspending the spores in water for 10 minutes at 58° C. Chemical activation was accomplished by heat-sensitizing the spores for 30 minutes at 46° C, as recommended by Sussman (**1954**), followed by treatment with furfural or furfural diacetate.

Spore concentrations were estimated from density readings made in a Klett colorimeter with a blue filter (Klett No. 42). In general, a final concentration of 1 mg dry weight per ml of spores was used. This corresponded to a Klett reading of 180.

Germination was studied by incubating 2 ml of a spore suspension, after the appropriate activation treatment, in a 25 ml Erlenmeyer flask on a reciprocal shaking machine at 30° C for three to four hours. At this time 5 drops of formalin were added in order to kill the spores, whereupon counts could be made. These were performed by a modification of the method of Davies, Duckworth and Harris (**1948**).

The treatment of dormant cells with enzymes was accomplished by incubating 1 ml of a spore suspension with an equal volume of the enzyme solution for 8 hours at 20° C. After such incubation the spores were centrifuged free of the enzyme solution in three changes of distilled water.

Lysozyme was obtained as the crystalline product from Armour and Company, Chicago, Illinois. Protease, pepsin, hemicellulase and hyaluronidase were furnished by Mann Research Laboratories, Inc., 136 Liberty Street, New York, while papain, lipase (steapsin) and pectinase were supplied by Nutritional Biochemicals Corp., Cleveland, Ohio. All enzyme solutions were prepared immediately before use and were never used after storage.

THE EFFECT OF LYSOZYME UPON DORMANT ASCOSPORES

Lysozyme was the first enzyme studied because of the large background of data that has been accumulated concerning its effect upon bacterial cells. Its ability to induce the lysis of these organisms was

first pointed out by Fleming (**1922**) who also recognized the enzyme's wide distribution in plants as well as in animals. Although the exact chemical nature of its substrate is poorly understood, it seems certain that the enzyme attacks a component of the cell wall (Weibull, **1953**; Oginsky and Umbreit, **1954**).

Suspensions of ascospores were incubated in 0.5% lysozyme for varying periods of time, after which the spores were washed free of the enzyme. Activation was effected by the means discussed in the section on METHODS and the spores incubated in distilled water or furfural solutions. As can be seen in TABLE I, lysozyme treatment for longer than

TABLE I

EFFECT OF LYSOZYME TREATMENT OF DORMANT ASCOSPORES UPON THEIR GERMINABILITY IN VARIOUS CONCENTRATIONS OF FURFURAL. THE ASCOSPORES WERE EITHER HEAT ACTIVATED OR CHEMICALLY ACTIVATED AFTER BEING HEAT SENSITIZED. RESULTS ARE THE AVERAGE OF TWO EXPERIMENTS. DASHES INDICATE NO MEASUREMENTS WERE MADE

Duration of lysozyme treatment (hrs.)	Percentage germination						
	Furfural-activated			Heat-activated			
	10^{-3} M	10^{-4} M	10^{-5} M	10^{-3} M	10^{-4} M	10^{-5} M	H_2O
0	80	83.2	10	88	89	89	86
1	75	74	12	85	86	86	85
2	40	46.6	23	81	86	88	83
3	50.1	52.8	3	66	68	90	90
4	38.2	30.1	1.7	42	79.1	61	49
6	11.7	16.4	0	14.2	4	14	42
8	7.8	—	0	11.3	—	0	31

8 hours drastically reduces germination of heat and chemically-activated ascospores when they are incubated in furfural. However, when spores are treated with lysozyme and then heat-activated and germinated in distilled water, the effect is quite variable and is not as marked as when furfural is added to the water in which the spores are germinated.

For the purpose of comparison, ascospores which had not been exposed to lysozyme were activated and incubated with the results shown in the same table. In these experiments it is clear that heat-activated ascospores are not significantly inhibited by furfural below a concentration of 1×10^{-2} M.

In the case of the furfural-activated ascospores the reduction in germination caused by lysozyme treatment (compare TABLE I) could have been due to either of the following reasons: an effect upon the response of ascospores to chemical activators; or, an altered permeability to

TABLE II

EFFECT OF LYSOZYME UPON ASCOSPORES WHICH WERE ACTIVATED IN 1×10^{-3} M FURFURAL AND WERE INCUBATED IN EITHER DISTILLED WATER OR IN FURFURAL. EXPERIMENTAL DETAILS AS DESCRIBED IN TEXT

Incubated in:	Percentage germination	
	Untreated	Lysozyme-treated
Distilled water	72	56
Furfural	70	16

furfural resulting in its accumulation in toxic amounts. This question was resolved by suspending lysozyme-treated ascospores in furfural for 10 minutes after which the chemical was removed by washing in 4 changes of distilled water. As is demonstrated in TABLE II, lysozyme-treated ascospores activated by short exposure to furfural germinate almost as well as the untreated control spores. By contrast, those incubated in furfural during the entire course of the germination period germinated very poorly.

Since the pH optimum for lysozyme activity differs depending upon the end-point used (Meyer, **1946**), the effect of this variable was studied. Ascospores were suspended in 0.5% lysozyme adjusted to pH 5.0, 6.3 and 7.3 with phosphate buffer. Treatment with the enzyme was continued for 14–16 hours after which the enzyme was removed as before. Activation was by both heat and chemical means, and spores receiving all treatments were incubated in two concentrations of furfural diacetate with the results shown in TABLE III. As a consequence, a pH of 6.3 and a treatment of 8 hours duration were routinely used unless otherwise indicated.

TABLE III

EFFECT OF PH UPON LYSOZYME TREATMENT OF ASCOSPORES INCUBATED IN DIFFERENT CONCENTRATIONS OF FURFURAL DIACETATE. ASCOSPORES WERE INCUBATED IN LYSOZYME FOR 14 HOURS

pH	Percentage germination			
	Furfural-activated		Heat-activated	
	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M
5.0	37	16.3	0*	11
6.3	0	0	0	0*
7.3	0	0*	0*	0*

* Although germ tubes had not extended, small blister-like protuberances were present at the ends of the spores.

The optimal concentration for lysozyme action was determined. The results are presented in FIG. 1 from which two observations are apparent: first, significant effects are obtainable at lysozyme concentrations as dilute as 1:12,800 (approximately 0.008%); and second, heat-activated ascospores suspended in furfural are almost equally sensitive

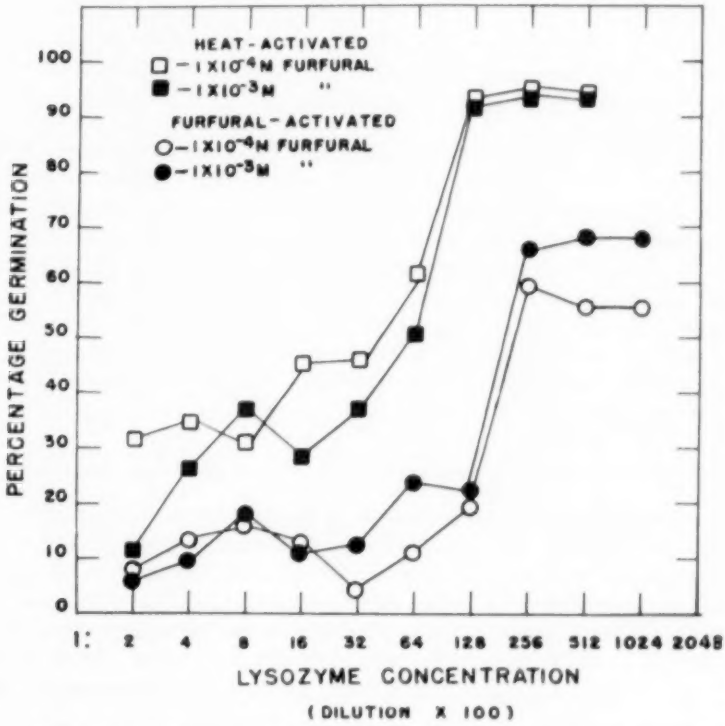

FIG. 1. Effect of dilutions of lysozyme upon the germination of *Neurospora* ascospores. Germination was accomplished in the concentrations of furfural shown in the legend. Lysozyme concentration is expressed as the ratio of enzyme to buffer (0.005M phosphate, pH 6.3). Ascospores were treated for 8 hrs.

to such treatment as are chemically-activated ones if allowance is made for the lower percentage of germination characteristic of the latter method of activation.

It was now possible to compare the action of lysozyme upon dormant and activated ascospores. Therefore, heat-activated spores were incu-

TABLE IV

EFFECT OF LYSOZYME TREATMENT UPON HEAT-ACTIVATED ASCOSPORES. DASHES INDICATE THAT NO COUNTS WERE MADE. FIGURES REPRESENT AVERAGE OF THREE EXPERIMENTS

Duration of treatment (hrs.)	Percentage germination	
	Lysozyme-treated	Water control
1	61.5	—
2	11.5	—
3	6.5	—
4	0	94.4

bated in 0.5% lysozyme and aliquots were withdrawn periodically and incubated, after washing in distilled water. The results given in TABLE IV show that only 3 to 4 hours of lysozyme treatment are required for complete inhibition of germination under these conditions.

Heat-sensitized ascospores were exposed to lysozyme for 4 hours and washed free of the enzyme. Furfural was then added and the percentage of germination was determined after incubation. TABLE V shows that, as in the case of dormant and activated cells (TABLE I and TABLE IV), lysozyme treatment decreases the ability of heat-sensitized spores to germinate in furfural.

THE REVERSIBILITY OF LYSOZYME INACTIVATION OF ASCOSPORES

It was of obvious importance to determine the locus of lysozyme's action in the cell. If, as was the case in Weibull's (**1953**) study with bacteria, the enzyme was acting on the cell wall, a change in the ascospore's permeability to water might be expected. Therefore, experiments were performed wherein lysozyme-treated ascospores were germinated in buffer as well as in water, with the results presented in TABLE VI. It can be seen that when ascospores are incubated in the

TABLE V

EFFECT OF LYSOZYME TREATMENT ON HEAT-SENSITIZED ASCOSPORES. FIGURES REPRESENT AVERAGE OF SIX EXPERIMENTS. ASCOSPORES WERE INCUBATED IN FURFURAL

Treatment:	Percentage germination in:		
	H_2O	1×10^{-3} M furfural	1×10^{-4} M furfural
Water control	1	90	74
Lysozyme-treated	0	10.3	4.5

presence of 0.05 M phosphate buffer their germinability is only slightly reduced by lysozyme treatment (compare with TABLE I). Such treatment has been carried out for as long as 24 hours, yet only a small decrease in the percentage germination resulted.

Similarly, if an organic buffer is used instead of phosphate, no effect of lysozyme treatment is apparent. This is demonstrated in TABLE VII wherein the results of an experiment using piperazine buffer are presented. The piperazine hydrochloride used was prepared by treating the hexahydrate salt with concentrated hydrochloric acid in the presence of 95% ethyl alcohol. The crystals were washed several times in ethanol and fresh solutions of the buffer were always prepared before use.

TABLE VI

EFFECT OF LYSOZYME TREATMENT OF DORMANT SPORES AND INCUBATION IN 0.05 M PHOSPHATE BUFFER, pH 6.3. RESULTS ARE AVERAGE OF TWO EXPERIMENTS

Duration of lysozyme treatment (hrs.)	Percentage germination						
	Furfural-activated			Heat-activated			
	10^{-3} M	10^{-4} M	10^{-5} M	10^{-3} M	10^{-4} M	10^{-5} M	H_2O
1	53	48	15	80	83	84	93
2	44	33	16	60	81	77	93
3	44	33	8	59	72	75	85
4	35	35	3	87	74	86	87
6	40	25	6	89	69	83	86
8	44	27	6	47	70	70	88
24	32	31	0	74	74	71	70

OCCURRENCE OF INSOLUBLE HEXOSAMINE DERIVATIVE(S) IN THE ASCOSPORE

Although the exact chemical nature of the substrate for lysozyme is not known, there seems to be agreement that it is of the nature of a mucopolysaccharide, that is, a hexosamine-containing polymer. It was of interest, therefore, to establish whether substances of this nature are present in the *Neurospora* ascospore. Consequently, analyses of total hexosamine and the amount found in the insoluble residue of the cell were run.[2] The method for total hexosamine consisted in hydrolyzing acetone-extracted and dried ascospores for 6 hours in sealed

[2] The authors are indebted to Dr. H. J. Blumenthal for performing these determinations and to Dr. S. Roseman for the use of the laboratory facilities of the Rackham Arthritis Unit of the University Hospital, University of Michigan.

TABLE VII

REVERSAL OF LYSOZYME INHIBITION BY PIPERAZINE-NaOH BUFFER, pH 6.1. FIGURES REPRESENT AVERAGE OF TWO EXPERIMENTS. ASCOSPORES WERE TREATED WITH LYSOZYME FOR 8 HOURS

Treatment	Percentage germination	
	Furfural-activated	Heat-activated
H_2O	—	64.0
1×10^{-2} M furfural	0	11.4
1×10^{-2} M furfural, 0.12 M piperazine buffer	29.5	90.5
1×10^{-2} M furfural, 0.06 M piperazine buffer	71.3	88.1
0.12 M piperazine buffer	—	81.2
0.06 M piperazine buffer	—	91.6

tubes containing 6 N hydrochloric acid at 100° C. Aliquots were then filtered and dried *in vacuo* to remove the acid, after which hexosamine was determined after acetylation with acetic anhydride (Roseman)[3] or by the acetylacetone technique recommended by Boas (**1953**). In both cases purification of the samples was accomplished by the use of a Dowex 50 column. The insoluble hexosamine residue was analyzed by the same means except that the soluble constituents were first extracted with 10% hot alkali followed by 2% hydrochloric acid. As a result, it can be seen in TABLE VIII that the ascospores contain about 1.7% total hexosamine and 1.42% in the insoluble cell residue. Whether the small amount of soluble hexosamine found is significant is not known.

EFFECT OF OTHER ENZYMES UPON ASCOSPORE GERMINATION

From the standpoint that the cell's outer surface may be a mosaic of chemical entities, it is conceivable that more than one component of

TABLE VIII

HEXOSAMINE CONTENT OF ASCOSPORES. METHODS OF DETERMINATION AS IN TEXT. PERCENTAGE HEXOSAMINE COMPUTED ON BASIS OF DRY WEIGHT OF ASCOSPORES

	Percentage hexosamine		
	Sample no. 1	Sample no. 2	Average
Total hexosamine	1.92	1.42	1.71
Insoluble hexosamine	1.55	1.28	1.42
Soluble hexosamine (by difference)	—	—	0.29

[3] S. Roseman, 1954. Unpublished data.

its surface is susceptible to enzymatic attack. Therefore, dormant ascospores were incubated for 16–18 hours in 1% solutions of various enzymes after which the cells were washed free of the enzymes, activated, and incubated as described previously for the lysozyme experiments. The enzyme solutions were buffered to the appropriate pH with 0.01 M phosphate, or with "tris" buffer (tris-hydroxyaminomethyl methane), and controls in the same buffer were run in parallel. The results given in TABLE IX show that at least one of the enzymes tried had a strongly

TABLE IX

EFFECT OF VARIOUS ENZYMES UPON GERMINATION OF ASCOSPORES. 1×10^{-3} M FURFURAL USED WHERE INDICATED IN TABLE. DASHES INDICATE NO RESULTS AVAILABLE

Enzyme	pH used	Percentage of control germination		
		Heat-activated		Furfural-activated
		Furfural	H_2O	
protease	5.0	95	96	100
	7.0	103	96	124
pepsin	3.5	100	100	100
	5.0	94	102	—
hemicellulase	5.0	96	100	178
	6.0	94	80	175
papain	5.0	23	29	16
	6.0	33	38	39
lipase (steapsin)	6.0	87	88	104
	7.0	94	94	94
	8.1	—	—	100
pectinase	5.0	101	102	87
	6.0	101	103	77
hyaluronidase	7.1	98	95	—

inhibitory effect upon the germination of ascospores. This was papain which was dialyzed overnight at 4° C before use. On the other hand, hemicellulase seemed to stimulate the germination of furfural-activated ascospores. It was concluded that none of the other enzymes tried showed a significant effect upon the ascospores.

DISCUSSION

These results suggest the possibility of the selective alteration of the ascospore surface by the action of lysozyme. Such an alteration is

reflected in the lessened resistance of dormant cells to low concentrations of furfural. Heat or chemically activated ascospores respond in the same manner so that significant decreases in the germinability of these cells occur after lysozyme treatment. On the other hand, the germination of heat-activated spores in distilled water was affected less than was germination of spores incubated in furfural. To the authors' knowledge, this is the first case wherein lysozyme is reported to act upon a fungus.

That susceptibility to lysozyme is not restricted to dormant cells was shown by the experiments wherein the germination of both heat-sensitized and heat-activated ascospores was decreased as a result of such treatment. In fact, the latter type of cell was more sensitive to this treatment, since complete inhibition resulted after only 3 hours of exposure to lysozyme (TABLE IV). This suggests the possibility that a change in the cell surface has resulted from activation, thereby rendering the ascospore more sensitive to enzymatic attack.

Several facts suggest that the effect of lysozyme is quite specific. First, lysozyme is obtainable as a crystalline material in highly purified form, so that the possibility of contaminants being responsible for the observed effect is remote. Moreover, the failure of the heated enzyme to act, as well as the inability of several other enzymes to markedly influence germination, indicates that the reaction is specific. In addition, the low concentration of lysozyme required to affect the cell (FIG. 1) suggests a specific reaction. On the other hand, it should be noted that lysozyme is a basic protein (Fromageot, **1948**) and that its effect might be due to electrostatic binding with the cell surface, in this way affecting its physical properties. However, it is believed that this can not be the sole explanation because unpublished experiments of the authors have shown that lysozyme acts differently from basic substances like protamine or polymyxin so far as its effect upon the binding of dyes like methylene blue is concerned.

The locus of action of the enzyme seems to be on the surface of the cell. Admittedly, mere size of the molecule is not enough to permit the assumption that a substance does not penetrate, but additional evidence buttresses this supposition. This evidence concerns the reversal of the lysozyme effect by incubation of the cells in phosphate or piperazine buffer. These experiments suggest that the ability of the cell to resist unfavorable osmotic environments is reduced by lysozyme and that favorable tonic conditions as, for example, buffer solutions, permit the cell to survive. This interpretation would be in keeping with that offered by Weibull (**1953**) who found that the protoplast of *Bacillus megaterium* could be maintained intact after lysozyme treatment only in

the presence of 0.02 M sucrose plus 0.03 M phosphate. Meyer (**1946**) has suggested that the primary mechanism leading to water imbibition and cellular disorganization consists of a depolymerization of a mucopolysaccharide that seems to be firmly bound to the cell surface. Although not enough is known about the constituents of the ascospore to permit conclusions to be drawn as to the chemical nature of the cell component affected by lysozyme treatment, the results in TABLE VIII show that a hexosamine-containing polymer, a suspected substrate of lysozyme, is present.

These results contribute somewhat toward an understanding of a possible physiological role for furfural. Such a role is suggested by the data showing that activated, lysozyme-treated ascospores are much more sensitive to 1×10^{-3} M furfural than are untreated cells. This fact, taken together with the ability of buffers to reverse this effect, suggests that furfural is involved in the water uptake of ascospores. Whether this explains the capacity of this chemical to activate ascospores is still not known. Finally, although the permeability of the cell to water is affected by lysozyme, activation does not occur. This is evidence that a change in permeability to water alone is not sufficient to break dormancy in ascospores of *N. tetrasperma*.

SUMMARY

Concentrations as low as 0.008% lysozyme drastically reduce the germinability of heat or chemically activated ascospores of *Neurospora tetrasperma* in solutions of furfural that are usually non-inhibitory. Various physiological states of the ascospore, that is, dormant, heat-sensitized and activated cells, are affected by the enzyme although the last-named are most sensitive. Phosphate and piperazine buffers reverse this effect in concentrations between 0.05 and 0.12 M. The crystalline nature of the enzyme, the failure of the boiled enzyme to act, and the small concentration needed indicate a specific enzymatic reaction. In addition, only papain, of seven other enzymes studied, showed an effect approaching that of lysozyme. These results are therefore consistent with the view that lysozyme alters the osmotic regulation of the cell, rendering it unable to survive unless it is suspended in the proper tonic environment. Finally, analysis shows that the ascospore has about 1.4% hexosamine in the insoluble cell residue (on a dry weight basis) as compared with a total hexosamine content of 1.7%.

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DEAMINATION AND DEGRADATION OF AMINO ACIDS BY STREPTOMYCETES

DAVID GOTTLIEB AND ORIO CIFERRI

Usually one strives to study the metabolism of streptomycetes in a medium as simple as possible; such conditions are not common in nature for these organisms generally live in the soil, where, in addition to inorganic compounds, many organic materials are available to them. It is therefore important to understand the metabolism of these organisms on organic substrata. Among such materials in soil are the amino acids which could furnish a source of nitrogen for the growth of these microorganisms. In order to get clues to the importance of these processes, studies were undertaken on the ability of the streptomycetes to deaminate these compounds and where possible, to determine the pathway of degradation.

Data on the nitrogen metabolism of the actinomycetes is restricted to relatively few papers. The early reports on this subject are represented by the first contributions of Krainsky (**12**) and Waksman (**17**, **18**) dealing with the culture, substrate utilization and pigment production of the actinomycetes. They furnished information concerning the ability of certain species to grow on media containing inorganic salts such as nitrates or ammoniacal salts or organic compounds such as urea, leucine, tyrosine, etc., as sole nitrogen source. In addition, the process of deamination of organic nitrogen compounds has been emphasized by Waksman as a means of measuring the growth of these organisms. Woodruff and Foster (**19**) show that *S. lavendulae* deaminates some amino acids but not others. Arginine, histidine, glycine and its homologues were all readily deaminated while certain other amino acids such as phenylalanine, the leucines, tryptophan, methionine, etc., were deaminated only slightly if at all. Other investigators showed the importance of amino acids as sole nitrogen sources for growth and antibiotic production (**2**, **5**, **7**, **13**) and pigment production (**3**). Dulaney (**5**) showed that *S. griseus* has very definite specificities for growth and streptomycin production; as sole nitrogen source, proline supported strikingly high production of antibiotic while the closely related glutamic acid, arginine and ornithine gave very poor yields.

MATERIALS AND METHODS

Cultures used in these experiments were *Streptomyces venezuelae R 1, S. lavendulae C–44, S. griseus C–1914* and *S. diastaticus*. All the amino acids were obtained from the Nutritional Biochemical Co. and were in the racemic form, except proline, phenylalanine and cysteine that were available only in the levo form. Sodium nitrate was Baker and Adamson's Reagent grade and urea was Mallinckrodt U.S.P.

The inoculum was prepared by transferring soil cultures to sterile slants of tryptone-glycerine agar (**8**) and incubating at 26° C for approximately one week. A spore suspension was made by adding 5 ml of sterile water to such slants and an aliquot was aseptically transferred to the most suitable medium for the growth of each streptomycete; *S. venezuelae* was grown, for instance, in the synthetic medium reported by Legator and Gottlieb (**14**), while the others were grown in Emerson's medium. The inoculum was grown in shake flasks until growth was sufficiently heavy, usually 48 hours. After this period the cells were harvested by centrifugation, washed and recentrifuged three times in 0.9% sodium chloride or phosphate buffer solution. The amount of cells present was determined as wet weight and the cells were finally suspended in known amounts of a similar sodium chloride solution. Equal weights of cells from such solutions were transferred to a series of triplicate 500 ml Erlenmeyer flasks, each containing single amino acids in solution at a concentration of 0.2 mg of nitrogen per ml. Any variation in the concentration due to the addition of the inoculum or to buffer was corrected for in the final calculation. Prior to inoculation, the flasks were sterilized by autoclaving at a pressure of 15 pounds for 20 minutes. The inoculated flasks were placed on a reciprocal shaker and held at 26° C.

When the amino acids were used as sole nitrogen source in a complete synthetic medium (**14**), the components of the medium and each amino acid were separately sterilized, each in double concentration, and were afterwards mixed aseptically. In these experiments the quantity of each amino acid was calculated and used in amounts necessary to give the same nitrogen concentration as was given by the sodium nitrate in the synthetic medium (0.3 mg of nitrogen per ml). Tryptophan, which in preliminary experiments was shown to be altered by the temperature of the autoclave, was sterilized by passing it through a sintered glass bacteriological filter.

Preliminary experiments showed the inadvisability of sterilizing amino acids and buffer together because of their interaction at the tem-

perature of sterilization. Therefore the amino acids and buffer solutions were sterilized separately and then mixed. If changes occurred in the pH of the buffer during sterilization, the solution was further adjusted and resterilized. After such a treatment the pH values varied no more than one tenth of a unit from the desired value. The buffer was a 0.1 molar solution of potassium dihydrogen phosphate and sodium hydroxide. Samples were taken at periods of time from 0 to 96 hours; they were filtered free of mycelium and frozen at $-40°$ C until analyzed.

The deaminated nitrogen was determined in some experiments by a semi-micro-Kjeldahl procedure. The results were found to be reliable and correlated well with the data obtained by direct nesslerization. The latter technique was preferred and normally used because of its speed and simplicity. The nesslerization was similar to the procedure of Morgulis and Spencer (**15**). Nessler's reagent was prepared according to the formula of Folin and Wu as reported by Hawk *et al.* (**9**). The colorimeter (Lumetron 400-A, Photovolt Co., N. Y.) was calibrated according to the directions given by the manufacturer (**16**). For the analysis, 0.5 ml aliquots of amino acid solution were transferred from the culture filtrates to calibrated tubes, diluted with water and the reagent then added. The intensity of color was read within 20 minutes on the colorimeter. A series of standard solutions of known concentration of ammonium sulfate were prepared and nesslerized to serve as standards. A 0.9% solution of sodium chloride, treated in the same manner as the other samples with a lumetron reading of 100% light transmission, served as a blank. Concentrations of the unknowns were calculated by interpolation on standard curves (percent of transmission *vs.* concentration on semilogarithmic paper) and calculated as percentage of available nitrogen. The sample taken at 0 hr constituted the first control; if for some reason, these samples showed values less than the blank, such readings were used to correct values at the subsequent periods. Three additional types of control were run in these experiments. A solution of sodium chloride inoculated with the test organism was used to ascertain the possible liberation of nitrogen by lysis of the mycelium or other metabolic changes occurring during the incubation period. An uninoculated solution of sodium chloride or of buffer was used to test the uptake of nitrogen from the atmosphere during the shaking period, since ammonia is used in the system of refrigeration of the laboratory. Finally, an uninoculated solution of sodium chloride containing any one of the amino acids (generally glycine) was used to ascertain the increase in the concentration of the amino acids due to the evaporation of the solution during the course of the experiment. In all cases the last three

kinds of controls showed that these factors did not influence the results.

The amount of growth was determined, after 72 hours on a reciprocal shaker, by filtering the mycelium on Whatman n.1 filter paper, washing three times with water and drying it at 80° C for 48 hr. After the mycelium had been dried, it was allowed to come to room temperature in a calcium chloride-containing desiccator. The final weights of mycelium were obtained by subtracting the weight of the filter paper and of the original inoculum from the total weight. Growth was expressed in terms of the weight increase over the initial amount of cells.

TABLE I

INFLUENCE OF THE pH ON DEAMINATION. DEAMINATED NITROGEN *

Amino acid	Time in hours	*S. venezuelae* pH					*S. lavendulae* pH				
		6.0 %	6.5 %	7.0 %	7.5 %	8.0 %	6.0 %	6.5 %	7.0 %	7.5 %	8.0 %
Glycine	12	0	0	0	0	0	0	0	3	0	0
	24	0	0	7	0	0	0	4	6	0	0
	48	0	15	18	8	4	3	16	16	10	9
	72	5	20	21	12	6	18	22	22	18	13
Alanine	12	0	0	0	0	0	0	0	0	0	0
	24	0	0	4	0	0	0	7	6	0	0
	48	12	12	15	21	8	0	10	7	9	5
	72	12	20	26	21	12	0	19	21	21	18
Arginine	12	0	0	0	0	0	14	13	16	20	15
	24	0	1	5	4	0	23	23	50	49	46
	48	0	5	17	10	13	45	49	52	51	46
	72	5	18	23	16	15	57	77	65	54	49
Histidine	12	0	0	0	0	0	0	0	0	0	0
	24	0	0	0	0	0	5	17	19	15	12
	48	0	4	14	19	5	40	78	53	42	37
	72	0	7	34	40	29	56	87	65	54	49
Glutamic acid	12	0	0	0	0	0	0	0	0	0	0
	24	0	17	7	0	0	5	8	6	0	0
	48	14	25	17	8	0	5	13	8	0	0
	72	15	29	20	14	0	12	31	23	8	0

* Each flask contained 0.19 mg of nitrogen per ml. The inoculum contained 0.12 gm of cells wet weight per flask. Buffer dispensed at the rate of 10 ml per flask. Each result is the average of three flasks.
Tryptophan was not deaminated by any of the tested organism at any tested pH.

The presence of various amino acids in the medium during metabolism was generally determined by two-dimensional ascending chromatography as described by Kirby-Berry *et al.* (11).

RESULTS

Deamination in buffered solutions: Studies in which the solutions were buffered at specific pH levels between 6.0 and 8.0 indicated that the optimum deamination occurs around neutrality (TABLE I). The organisms were unable to deaminate glutamic acid and arginine in unbuffered solutions, for these amino acids changed the solutions to pH 3.7 and 10.5 respectively, thus preventing enzyme action. No great differences were found in the case of glycine and alanine in buffered and unbuffered solutions since their reactions are normally around neutrality. However, certain amino acids such as tryptophan could not be deaminated under any conditions which were used in this study. Apparently this was related to their inability to support growth, as will be shown later. At pH 6.9 (TABLE II) *S. venezuelae* consistently deaminated nine amino acids (glycine, alanine, serine, threonine, glutamic acid, arginine, histidine, phenylalanine and proline); *S. lavendulae* deaminated the same amino acids with the exception of phenylalanine. Deamination was usually slow but *S. venezuelae* deaminated most of these compounds more quickly and in greater amounts than did *S. lavendulae*. With *S. venezuelae* no consistent deamination occurred for more than 24 hours after inoculation, except with glutamic acid and arginine; *S. lavendulae* did not deaminate any of the amino acids in the first 12 hours and

TABLE II

RATE OF DEAMINATION AT pH 6.9. NITROGEN DEAMINATED *

Organism	Time in hours	Amino acids**								
		Gly. C_4	Ala. C_3	Ser. C_3	Thr. C_4	Glu. C_5	Arg. C_6	His. C_6	Phe. C_9	Pro. C_5
S. venezuelae	12	0	5	6	5	25	22	0	0	5
	24	16	14	12	16	34	25	9	7	10
	48	22	38	25	22	44	32	21	9	28
	72	38	38	32	25	48	46	65	14	46
S. lavendulae	12	0	0	0	0	0	0	0	0	0
	24	0	3	0	0	0	8	22	0	0
	48	16	6	0	0	5	38	43	0	6
	72	36	17	12	9	20	52	52	0	10

* Each flask contained 0.18 mg of nitrogen per ml. The inoculum contained 0.12 gm of cells wet weight per flask. Each result is the average of three flasks.

** The following amino acids have not been deaminated by any of the organisms: cysteine, tyrosine, leucine, norleucine and tryptophan.

The following abbreviations have been used in this table: Gly-glycine, Ala-alanine, Ser-serine, Thr-threonine, Glu-glutamic acid, His-histidine, Phe-phenylalanine, Pro-proline.

achieved appreciable amounts of deamination only after 48, or even 72 hours.

The two organisms showed a consistent difference in their ability to deaminate phenylalanine. The fact that this amino acid is deaminated only by *S. venezuelae* could be explained by a particular role that phenylalanine might play in the metabolism of this streptomycete (**6**). Another interesting feature is the extreme structural specificity of deamination; tyrosine (which differs from phenylalanine only in the presence of an hydroxyl group in the *para* position) was not deaminated by this species.

Pathways of deamination of histidine and arginine: The ability of these microorganisms to break down amino acids with complex structures, such as arginine, histidine and proline, compared with the inability to attack others with linear structures, such as the leucines, might suggest that a simple cleavage from the carboxyl end of the chain as in fatty acids does not occur.

Preliminary studies on these pathways of deamination by *S. venezuelae* were made using paper strip chromatography. The brews containing histidine were chromatographed at intervals from 0 to 120 hours. In the first 48 hours there was only one ninhydrin-positive spot with an Rf of 0.63 in phenol that corresponded to histidine. Samples taken at 72 and 96 hours showed, in addition, another spot with an Rf of 0.23 that corresponded to the Rf of 0.24 given by glutamic acid in a mixture of pure glutamic acid and histidine that was run concurrently. The sample taken at 120 hr did not show any ninhydrin-positive reaction, thus pointing out the disappearance of the amino acids in the range of sensitivity of the test.

An analysis of the deamination pattern of arginine showed only one spot corresponding to this amino acid through 72 hours. After 96 hrs another spot appeared, corresponding to ornithine. All attempts to detect the presence of urea by the method reported by Kirby-Berry (**10**) were unsuccessful.

Amino acids as sole nitrogen source: Single amino acids were substituted for sodium nitrate in a complete synthetic medium (**14**) and the extent of growth was determined. The results (TABLE III) showed clearly that the amino acids deaminated in pH 6.9 buffer supported good growth in synthetic medium, while those that were not deaminated supported at best a scanty growth. The increases in weight with the non-deaminated amino acids were not more than 8 times the inoculum while cells grown in a synthetic medium, without any nitrogen source, resulted in a 6-fold weight increase. On the other hand, the amino acids

which could have been deaminated allowed growth ranging from 14 to 22 times the initial weight. Alanine, glycine and histidine permitted excellent growth, even higher than a sodium nitrate-containing medium. Serine, arginine, proline and glutamic acid supported good growth. Tryptophan, leucine and norleucine, amino acids that were not deaminated, resulted in extremely poor growth. Phenylalanine was unusual because it could be deaminated but was unable to support growth; however, its degree of deamination is small compared with that of any other of the deaminated amino acids.

TABLE III

GROWTH OF *S. venezuelae* IN THE PRESENCE OF AMINO ACIDS AS SOLE NITROGEN SOURCE

Nitrogen source*	Nitrogen deaminated at 72 hours (Data from TABLE III) %	Growth Increase over inoculum** gm
Urea	—	.108
No nitrogen	—	.128
Phenylalanine	14	.146
Norleucine	0	.160
Tryptophan	0	.161
Leucine	0	.177
Glutamic acid	48	.298
Proline	46	.303
Arginine	46	.357
Serine	32	.382
Sodium nitrate	—	.427
Histidine	65	.449
Alanine	38	.458
Glycine	38	.463

* Each flask contained 0.3 mg of nitrogen per ml. Each result is the average of three flasks.

** Inoculum 25 mg of cells dry weight per flask, subtracted from the reported values.

DISCUSSION AND CONCLUSIONS

Streptomyces venezuelae is able to deaminate many amino acids; of the fourteen tested in the present experiments, nine have been consistently cleaved in this manner. Of these nine, some, such as glycine and homologues, have very short and simple chains, while others such as glutamic acid and arginine, have more complex structures, or, such as histidine, proline and phenylalanine, even contain aromatic rings. This organism is very specific in its ability to deaminate; for example, the —CH—NH_2 bond is broken down in glycine and related compounds but not in the leucines; the nitrogen-containing rings of histidine and proline are cleaved and deaminated but not the ring of tryptophan. Even more striking is the fact that phenylalanine is deaminated while a close analogue, tyrosine, is not attacked. The products of the deamination are

not detectable until 12 hrs after inoculation; the ammonia liberated increases after 24 hrs and reaches a plateau between 48 and 72 hrs, the period in which growth is at a maximum.

The deamination is affected by the pH of the medium; its optimum is around neutrality and too acid or too basic conditions completely inhibit it. Neutral amino acids are easily deaminated, even in unbuffered media, while acidic or basic ones require buffering in order to be deaminated. Basic amino acids are frequently not deaminated if the medium is even slightly acid and vice versa: *S. venezuelae* does not deaminate arginine at pH 6.0 nor glutamic acid at pH 8.0.

The simpler amino acids are presumably deaminated by a cleavage involving only one reaction; more complex ones might require more than one step and more than one deamination. Determination of amino acids by paper chromatography indicated that histidine was transformed into glutamic acid, perhaps via the classical degradation pathway of urocanic acid, and glutamic acid, in turn, was deaminated to alpha-keto glutaric acid. This pathway seems to be in agreement with the quantitative results obtained, especially in one of the deamination experiments (TABLE II), but does not as readily fit the data in TABLE I. In the first 48 hours only 21% of the total nitrogen had been deaminated. Since each of the three nitrogens of histidine accounts for 33% of the total nitrogen, it may be supposed that in this first period substantially one cleavage had occurred: the transformation of histidine to urocanic acid. This would explain why the chromatograms made during the 48 hr period showed only the histidine spot. In a second period from 48 to 72 hrs and over, the other two degradations (the cleavage of the imidazole nucleus and the deamination of glutamic acid) occurred. At 72 hrs, 65% of the total available nitrogen had been deaminated, a quantity that could correspond to 100% deamination of two of the three nitrogens. But since, in the case of all the other amino acids, not more than 40 or 50% of the total nitrogen present had been deaminated, it might be concluded that all the three nitrogen groups of histidine had been cleaved from the molecule. This conclusion is also suggested by the fact that the chromatogram prepared after 72 hours still showed a spot of undegraded histidine, and that even glutamic acid alone, that is, the last amino acid appearing during histidine degradation, was deaminated at 72 hours only to the extent of one-half of the total nitrogen available.

Paper chromatograms also indicated that the pathway of dissimilation of arginine may be similar to that found for many species of streptococci leading to formation of one mole of carbon dioxide, two moles

of ammonia and one mole of ornithine. This hypothesis is in agreement with the percent of deamination. Since only two of the four amino groups of arginine were deaminated in such a system, the amount of nitrogen released should not have been higher than 50% of the total nitrogen present. As a matter of fact the experimental results showed 46% of the total nitrogen deaminated after 72 hours.

The amino acids easily deaminated by *S. venezuelae* are those directly or indirectly involved with the citric acid cycle. This would be in agreement with the results of Cochrane (**1**) and Cochrane and Peck (**4**) on the presence of such a cycle in certain species of streptomycetes. In addition, experiments in progress at present in this laboratory indicate a linkage between deamination (or its reversal) and the synthesis of the amino acids in the cells. *S. venezuelae* cells, grown in a synthetic medium containing norleucine-2-C14, produced radioactive acetate in the brew and radioactivity was found in the mycelial protein. The major part of such radioactivity was found in five or six amino acids, namely glutamic acid, aspartic acid, proline, arginine and one or more of the leucines. These results suggest that norleucine is cleaved to acetate and then converted to "active acetate." The acetate goes through the citric acid cycle to alpha-keto glutaric acid and succinic acid which in turn are used for the synthesis of new amino acids.

With the exception of phenylalanine, *S. venezuelae* and *S. lavendulae* in buffered media deaminate the same amino acids. The results for *S. lavendulae* are in agreement with those reported by Woodruff and Foster (**19**) although in the present study a different strain was used. The amino acids that can be deaminated in buffer solution are those that are able to support the growth of *S. venezuelae* when used as sole nitrogen source in a synthetic medium. This suggests a close correlation between growth and deamination; the organism grows utilizing the carbon skeleton that is set free from the deaminated amino acids. The striking stimulatory effect on growth of glycine and alanine may be explained by the easy availability of the carbon skeleton of these compounds for the formation of acetates and the synthesis of carbohydrates, proteins and fats.

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SUMMARY

1. *S. venezuelae*, in a saline solution buffered at pH 6.9, deaminated glycine, alanine, serine, threonine, glutamic acid, histidine, arginine,

proline and phenylalanine. Under these conditions the organism was not able to deaminate tyrosine, leucine, norleucine, cysteine or tryptophan. A similar pattern was shown by *S. lavendulae* for all the amino acids but phenylalanine.

2. The deamination was influenced by the pH of the solution with an optimum near neutrality. The deamination of acidic amino acids was easily inhibited in slightly basic solutions and that of basic amino acids in acidic solutions.

3. The pathway of dissimilation of histidine by *S. venezuelae* was through glutamic acid and of arginine through ornithine.

4. The amino acids that were deaminated by *S. venezuelae* in pH 6.9 buffer were the same ones that were able to support growth when used as sole nitrogen sources in a synthetic medium. The amino acids that were not deaminated were not able to support growth.

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HISTOPLASMA DUBOISII AND LARGE FORMS OF HISTOPLASMA CAPSULATUM

R. VANBREUSEGHEM

The description of a new species, particularly in the field of mycology, is usually accepted without concern, if not with a cold smile. If such species does not afford anything of general interest, it is soon forgotten. If it happens to be close to some important section of taxonomy or pathology, every effort is made to have it accepted as a synonym or, for the most generous, as a variant.

From personal communications, from private correspondence and from a recent publication I suspect that such could be the lot of *Histoplasma duboisii* Vanbreuseghem 1952 (**8**, **23**). In a recent paper, Van Uden, Chaves de Almeida and Singer (**25**, 1954) wrote without demur, "In pathological material like pus and sputum, the yeast-like cells of *H. capsulatum* may be extracellular and much bigger than usual (so-called *duboisii* form of Vanbreuseghem). . . ." If the first part of this sentence is quite correct, the phrase in parentheses appears to me unsound.

Have we any reason to think that the *duboisii* forms of *H. duboisii* are merely large forms of *H. capsulatum?* And if such is the case, should we consider the first a synonym of the second? Or, on the contrary, have we any reason to consider *H. duboisii* as a species which is very near to but different from *H. capsulatum?* This last point of view is mine and I am afraid that for the present a different point of view cannot be supported.

Histoplasma duboisii was isolated for the first time from a Belgian patient who had returned from the Belgian Congo (Dubois, Janssens and Brutsaert, **8**). Later, Dubois and I (**9**) described from eight earlier presumed cases of histoplasmosis (**3**, **4**, **10**, **11**, **14**, **15**)[1] from Africa an African histoplasmosis whose existence had already been suspected by myself (**22**). A new case of African histoplasmosis was published in 1953 by Clarke, Walker and Winston (**5**). Two other cases were published in 1954 by Silveira and Konstam (**21**) and by Audebaud, Merveille and Depoux (**1**). The conclusion of Audebaud *et al.* was that African histoplasmosis was a distinct entity. It is nevertheless fair to

[1] Also J. T. Duncan, personal communication (see Ref. **9**).

add that the fungus was not isolated from the patient of Silveira and Konstam (**21**) and that the mycological diagnosis made by J. Walker had no more than a histological basis.[2]

The description of *Histoplasma duboisii* as a new species (**8**) is founded on the morphology of this fungus in the parasitic state. It is a large, budding, ovoid cell, with a thick membrane, and it contains one or more droplets of fat. At first, the parasite is entirely intracellular; later on, the parasites are found in the pus and at this stage their morphology is very irregular (**23**). In the guinea pig, 8 to 15 days after intratesticular inoculation, the parasites look so much like *Histoplasma capsulatum* that I named these forms the *capsulatum* form of *Histoplasma duboisii*. Later, the parasites enlarge and their mature forms are what I have named the *duboisii* form of *Histoplasma duboisii*. In man, the *duboisii* form is the only one to have been discovered thus far; this is a point of importance in considering the fact that in human cases, where large forms of *H. capsulatum* have been found, these large forms are always exceptional, and most of the parasites display the classical morphology accepted for *H. capsulatum*.

In culture, the mycelial phase of *H. duboisii* is very similar to the mycelial phase of *H. capsulatum*. Nevertheless, I have observed that whereas in *H. capsulatum* the macroconidia are both round and pyriform, in *H. duboisii* no pyriform macroconidia exist.

The yeast phase of *H. duboisii* is a mixture of small and large yeast cells. When the yeast phase of *H. capsulatum* or of *H. duboisii* is ageing, larger cells are noticeable in both species, but they are always larger in the latter than in the former species. This is also of importance in understanding why, in some cases, large forms of *H. capsulatum* do appear in the tissues.

The large forms of *H. capsulatum* were noticed for the first time by Crumrine and Kessel in 1931 (**6**) in the spleen of a 42-year-old man. The postmortem diagnosis was: histoplasmosis and intracellular parasites typical for *H. capsulatum* were found in sections, but larger, extracellular forms were also found in the spleen. Of course, no cultures were made, since De Monbreun had not yet published the demonstration of the fungal nature of *H. capsulatum* (**7**).

In 1953, Schwarz, in his interesting paper on giant forms of *H. capsulatum* in tissue explants, reported on the presence of similar forms in a caseating adrenal gland. These forms had been accepted by well-known mycologists as *Blastomyces* or *Candida* but, says Schwarz, "con-

[2] Personal communication.

sidering the observations presented herein, such forms may represent *H. capsulatum* similar to those described in the tissue explants."

Recently Binford (**2**) reported having found large forms of *H. capsulatum*, extracellular, in necrotic adrenal glands, in a necrotic renal medulla, and on valvular lesions. Whereas in the living tissues *H. capsulatum* was present in its normal, small and intracellular form, the large forms were more and more numerous in proportion as the tissues were more necrotic. The same author found in necrotic tissues some septate hyphae from 15 to 20 μ long. Another author, Humphrey (**13**), had, in 1940, already observed in a pulmonary cavity a tangle of mycelial threads. Although it was a case of disseminated histoplasmosis, Humphrey reached no definite conclusions as to the nature of these hyphae. In 1952, Haley (**12**) discovered in the necrotic liver of a mouse infected with the yeast phase of *H. capsulatum* tuberculated chlamydospores and hyphae. It is probably of lesser interest to note that in animals infected with the mycelial phase of *H. capsulatum* or of *H. duboisii*, tuberculated chlamydospores may be found for a long time after the inoculations.

In short, we may accept the conclusion that the finding of large forms of *H. capsulatum in vivo* is very rare. But there are also some reports of large forms *in vitro*. The first report was made in 1934 by De Monbreun (**7**), who observed that in the colonies on blood agar slants at 37° C more than a week old, some yeast cells of *H. capsulatum* were from two to three times larger than the normal yeast cells. Schwarz, in tissue explants from human cases or from animals, observed and described giant forms of *H. capsulatum*. These giant forms developed at 37° C in tissue explants on blood agar, not only in the lymph nodes excised from human patients, but also in the liver and spleen of mice infected with *H. capsulatum*. These giant forms may attain 20 μ in 2 to 3 weeks. Schwarz believes that the giant forms are "simply a growth in size of the original small yeast-like cells. We are not dealing with chlamydospores borne on hyphae because the increase in size of the cells is observed before hyphae appear."

On the basis of the studies heretofore reported we may admit: 1. That in tissues infected by *H. capsulatum*, large forms which may reasonably be attributed to that fungus occasionally appear. 2. That similar forms may develop in tissue explants or in aged colonies of the yeast phase of *H. capsulatum* at 37° C. 3. That *in vivo* these large forms are always extracellular. *They develop in necrotic, caseating tissues which probably reproduce the conditions in which the yeast phase grows in vitro.*

Binford (2), making a comparison between his own observations and the experimental work of Schwarz, admits that "it is conceivable that in the vascular thrombi and in the tip of the necrotic renal papilla, some of the conditions of tissue culture have been duplicated. The fungus had been continually exposed to a liquid medium, blood and urine. In these cases the atypical forms were most prevalent in the sites most distant from the viable tissues."

Moreover, we cannot forget that recently a large variability in the size of fungi in the parasitic state has been observed by different authors (16, 17, 19, 20, 21, 27). The variability in the morphology of the same fungus is a well-known fact, and I have thought it would be useful that such terms as parasitic state, saprophytic state, mycelial phase, yeast phase and form be properly defined (24).

In conclusion, I think it safe to say that *Histoplasma duboisii* is not a mere aspect of *Histoplasma capsulatum* and that the so-called large forms of *H. capsulatum* are not related to *H. duboisii.*

1. The *duboisii* form of *Histoplasma duboisii* in man is the only recognized parasitic form of *H. duboisii.* This parasitic form is also the only one to be found in the animal some weeks after inoculation.

2. In living tissues, the *duboisii* form is always intracellular. On the contrary, the so-called large forms of *H. capsulatum* are extracellular and to be found only in necrotic tissue.

3. In the guinea pig, during the first weeks after intratesticular inoculation, there exists a form of *H. duboisii* which is very similar to the parasitic state of *H. capsulatum:* that is why I named it the *capsulatum* form of *H. duboisii.* But in some weeks time this *capsulatum* form disappears completely and the *duboisii* form is the only one to be found.

4. A comparison between the *duboisii* form of *H. duboisii* and the so-called large forms of *H. capsulatum* shows readily that these cells have more differences than similarities. Of course, both of them are large cells, but whereas the *duboisii* forms are nearly always ovoid, with a very regular, double-contoured membrane, the large forms of *H. capsulatum* are quite irregular and are rarely or never ovoid (see Schwarz, 18 and Binford, 2).

5. In necrotic tissues of animals infected by *H. duboisii,* some extracellular forms develop; they are larger than the intracellular forms and much larger than the extracellular large forms of *H. capsulatum.* Both parasites behave similarly in similar conditions.

6. In cultures of *H. duboisii,* maintained at 37° C to obtain the yeast

phase, large forms develop, but they are always larger than the large forms produced by *H. capsulatum* under the same conditions.

SUMMARY

We might admit that *H. duboisii* is a giant type of *H. capsulatum*, but there are no reasons to regard *H. duboisii* as nothing more than the large forms of *H. capsulatum* recently described in necrotic tissues and in tissue explants. Both parasites have their individuality and so far as we can judge *H. duboisii* is a species distinct from *H. capsulatum*.

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ADDITIONS TO THE PHYCOMYCETE FLORA OF THE DOUGLAS LAKE REGION. II.[1] NEW CHYTRIDIACEOUS FUNGI

R. A. Paterson [2]

(with 2 figures)

During the summer of 1954, an investigation of chytridiaceous fungi was carried on in the Douglas Lake Region, Cheboygan County, Michigan. In addition to the numerous well-known forms encountered in the course of this work, two new species and one hitherto unreported in the United States were found. Two of the fungi were parasites of phytoplankters collected in Douglas Lake; the other occurred abundantly on representatives of several genera of green and blue-green algae in the vicinity of the University of Michigan Biological Station.

Phlyctochytrium unispinum sp. nov. (Fig. 1, a–i)

Sporangium sessile, membrana ex parte formata zoosporis separatis, dimidio quoque hemisphaerico, toto aliquando plus minusve ellipsoideo, 12.5–17.5 μ in diametro, apice maturo uno, 3.5–6.5 μ longo; systemate endobiotico praedito sphaerica apophyse 7–21 μ in diametro; systemate rhizoidali singulo et isodiametrico, 0.5–1 μ lato. Zoosporae sphaericae, 6–7.5 μ in diametro, singulo eccentrico globulo oleari, emissae per basim spinae in vesiculam tumentis, terminali parte spinae non soluta et liberata. Sporae perdurantes non observatae.

Sporangium sessile, the wall in part formed by the separation of the zoospore cyst, each half of which remains an unmodified hemispherical projection, giving the whole a somewhat irregular ellipsoid appearance, 12.5–17.5 μ from one portion of the zoospore cyst wall to the opposite wall, bearing at maturity a single apical spine, 3.5–6.5 μ long; endobiotic system a spherical apophysis 7–21 μ in diameter with a single isodiametric rhizoid, 0.5–1 μ wide; zoospores spherical, 6–7.5 μ in diameter, with a single eccentric oil globule surrounded by granular protoplasm, discharged into the basal portion of the spine which swells into a vesicle, liberated upon the dissolution of the vesicle, leaving the terminal part of the spine undissolved and free in the medium; resting spore not observed.

[1] Additions to the Phycomycete Flora of the Douglas Lake Region. I. F. K. Sparrow and M. E. Barr, Mycologia **47**: 546–556. 1955.

[2] Contribution No. 1041 from the Department of Botany, University of Michigan, and from the University of Michigan Biological Station.

Saprophytic on *Oedogonium* sp., *Zygnema* sp., and *Stigonema* sp., Nichols' Bog, Smith's Bog, Cheboygan Co., Michigan, June 28, 1954.

The first of the two new species was found on moribund and decaying plants of *Oedogonium* sp., *Zygnema* sp., and *Stigonema* sp. in

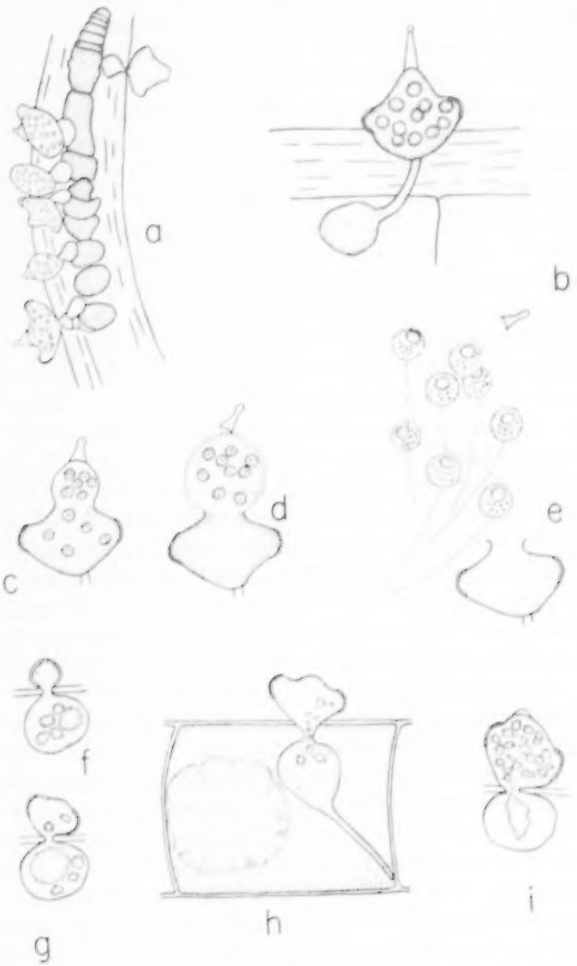

FIG. 1. a–i. *Phlyctochytrium unispinum*. a. Sporangia on filament of *Stigonema* sp. b–e. Stages in the discharge of zoospores. f–i. Stages in the development of the sporangium on *Zygnema* sp. a, × 473; all others, × 1147.

Nichols' and Smith's bogs, Cheboygan County. Two unusual features which characterize it are the peculiar sporangial development and the presence of an apical spine on the sporangium.

The method of development of this chytrid is as follows: The spherical quiescent zoospore, bearing a single oil globule and at rest on the substratum, soon becomes invested with a thick wall. Upon germination a coarse germ tube (0.5 to 1.0 μ in diameter) is produced, whose distal part within the substratum gradually expands into an apophysis. This germ tube often remains as a long connection (up to 9 μ) between the sporangium and apophysis, with the latter deep in the substrate (FIG. 1, b), or it may be short when the apophysis is just inside the host cell wall (FIG. 1, f). In *Stigonema* the apophysis is often found formed in the thick gelatinous sheath of the alga, the germ tube apparently not penetrating the host cell proper. As further development of the thallus continues the apophysis becomes filled either with numerous small oil droplets or with a single large translucent globule. Some of this material is undoubtedly contributed by the zoospore cyst, since the oil droplets can readily be observed migrating from it into the apophysis. The unexpanded cyst is ultimately devoid of oily material. At the time the apophysis reaches full size (7.0 to 21 μ in diameter) there occurs a vertical separation of the organized wall of the cyst into two halves. During the ensuing expansion of the epibiotic part, the cyst wall halves are retained as lateral projections of the developing sporangium. Shortly after this separation of the cyst wall the contents of the apophysis may be seen to move into the developing zoosporangium. It is probable, however, that actual movement occurred at the time of separation and that the increased volume of material flowing in caused the splitting. At the base of the apophysis there is produced a single unbranched rhizoid (0.5 to 1.0 μ in diameter) which does not appear to taper. When the sporangium has reached its maximum size, a knob-like projection is then formed between the old cyst walls. This knob, which is of a gelatinous consistency, gradually elongates to form a spine. The contents of the zoosporangium are at first granular, interspersed with fine oil droplets and these eventually coalesce in typical chytridiaceous fashion to form the oil globules of the zoospores.

Zoospore discharge is initiated by a flowing of the sporangial protoplasm into the basal portion of the spine, which in turn gradually expands into a vesicle, leaving the terminal portion intact. Delimitation of the zoospores is not completed until just before deliquescence of the vesicle wall although the oil globules are formed before the protoplasm is extruded into the spine. After migration into the vesicle the oil

globules decrease slightly in size. Prior to the deliquescence of the vesicle wall the zoospores begin to move about, and when the wall is gone they remain in an oscillating cluster for a few seconds before separating and swimming away. The spherical zoospores, 6.0 to 7.5 μ in diameter, are posteriorly uniflagellate and contain a single eccentric colorless oil globule surrounded by numerous granules. Of special interest here is the fact that the spine persists after discharge and is not wholly used in the production of the vesicle.

Although resembling somewhat an operculum the spine is not considered here such a structure. Its gelatinous consistency resembles, rather, the clear and refractive material of a discharge papilla of an inoperculate chytrid. An operculum, on the other hand, is not of a gelatinous nature, but rather appears generally to be of the same material as the sporangium wall. The spine, therefore, seems to be a highly developed discharge papilla in which the terminal portion does not enter into vesicle formation. This condition, in which part of the papilla does not dissolve, has been found by Sparrow (**1943**) in *Diplophlyctis laevis* Sparrow, in which he observed distinct remains of the discharge papilla on the surface of the zoospore mass after discharge. In operculate chytrids the operculum is pushed up or aside and may remain attached to the sporangium. The terminus of the spine in *Phlyctochytrium unispinum* is not displaced by the zoospores since they extrude only into the basal portion, apparently not requiring the total space offered by the spine. The terminal portion is not forcibly pushed away but merely becomes detached after the wall of the basal portion has deliquesced.

This fungus is placed in the genus *Phlyctochytrium* because of its inoperculate sporangium, its endobiotic apophysis, and its discharge by means of an apical papilla. A revision of its position may be necessary when the resting spore is observed. The apical spine in *Phlyctochytrium unispinum* superficially resembles that found in *Obelidium*. The methods of development and zoospore discharge are, however, entirely different. In *Obelidium* the spine is early differentiated from the body of the encysted zoospore and discharge is effected through a lateral pore. *Phlyctochytrium unispinum* most closely resembles *P. mucronatum* Canter in its possession of an apophysis, lateral projections, and apical spine. However, in the latter fungus, the sporangium does not develop by means of zoospore cyst separation as in *P. unispinum*. Furthermore, its zoospore discharge is by means of a lateral pore, not by extrusion into the apical spine. Other differences such as sizes of apophysis, nature of rhizoidal system, and sizes of zoospores separate the two. The unique method of discharge and the unusual development

of the sporangium clearly set off *P. unispinum* from other species of *Phlyctochytrium*.

Rhizophydium pedicellatum sp. nov. (FIG. 2, a–e)

Sporangium ovatum vel clavatum, leve, 9–14.5 μ longum, 7–12 μ crassum, stipite extramatricali 3–9 μ longo, plerumque procumbens, stipite parallelo vel recto, plus minusve perpendiculari filo; systema rhizoidale crassum, ramosum, gradatim minus; zoosporae numerosae sphaericae, 2–4 μ in diametro, globulo refractivo et singulario, 1–2 μ in diametro, granulis minutis et obscuris, flagello posteriore 15 μ longo praeditae; ex uno poro apicali emittentes. Sporae perdurantes non observatae.

Sporangium clavate to ovate, smooth walled, 9–14.5 μ high by 7–12 μ in diameter, borne on an extramatrical stalk, 3–9 μ long, usually procumbent, with the main axis and stalk parallel with the host, or erect, with the stalk more or less perpendicular to the host filament; rhizoids coarse, branched and tapering; zoospores numerous, spherical, 2–4 μ in diameter with a single eccentric refractive globule, 1–2 μ in diameter, a small dark granule, and a posterior flagellum 15 μ long, escaping through a single apical pore; resting spore not observed.

Parasitic on *Melosira italica* in plankton from South Fish Tail Bay, Douglas Lake, Cheboygan County, Michigan, August 20 through August 31, 1954.

During the last two weeks in August, 1954, a chytridiaceous parasite was found growing on the planktonic diatom, *Melosira italica*, in Douglas Lake. Although no resting spores were observed other features marked it as a new species of *Rhizophydium*, which has been termed *Rhizophydium pedicellatum*.

The typical pattern of development is as follows. After the zoospore has encysted on the host cell a germ tube is sent out laterally which grows for some distance (up to 9 μ) before it penetrates the siliceous cell wall of the diatom. There appears to be no specificity as to the place of penetration. It is possible that entrance is gained through the areolae. After penetrating the cell the extramatrical part of the germ tube expands and elongates into a clavate stalk and the internal part ramifies to form a much branched rhizoidal system. Often a small rhizoid-like branched structure which projects into the medium is found growing out of the stalk. The rhizoids penetrate the chromatophores, eventually causing them to shrink and turn brown. Following penetration of the germ tube and establishment of the endobiotic system the encysted zoospore begins to enlarge into a clavate or ovate sporangium (7 to 12 μ wide by 9 to 14.5 μ long). The single oil globule enlarges and splits up into many irregular shaped refractive globules of different

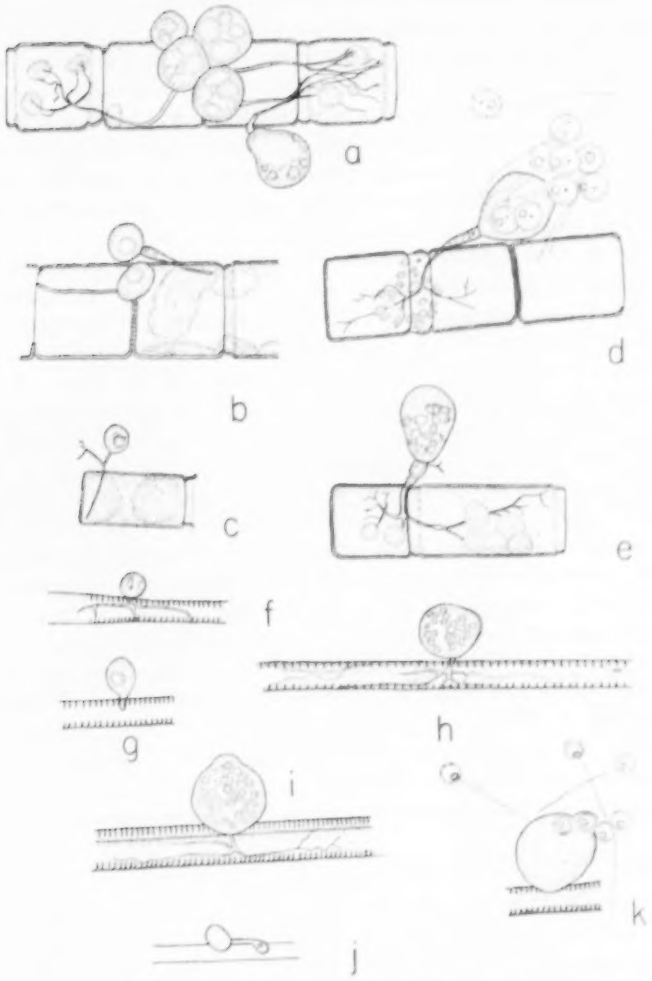

FIG. 2. a–e. *Rhizophydium pedicellatum* on *Melosira italica*. a. Sporangia on host. b, c. Developing sporangia. d. Sporangium with discharging zoospores. e. Sporangium on host. f–k. *Zygorhizidium planktonicum* Canter on *Synedra* sp. f, g. Developing sporangia on host. h, i. Sporangia. j. Sexual stage with conjugation tube. k. Discharging sporangium. k. Free hand, approx. × 470, all others, × 1147.

sizes. Before formation of the zoospores these small globules aggregate, become spherical and of nearly uniform size (1 to 2 μ). The zoospores are then delimited and at maturity the apical papilla dissolves. Discharge may be *en masse*, with the zoospores beginning to oscillate after a few seconds, or there may be a rapid swarming inside the sporangium after which they are liberated individually through the apical pore. They are 2 to 4 μ in diameter, spherical, with an eccentric oil globule (1 to 2 μ), one small highly refractive granule, and a posterior flagellum, 14 to 15 μ long.

The clavate stalk of *Rhizophydium pedicellatum* very closely resembles the broad swollen rhizoid of *Rhizophydium digitatum* Scherffel and the clavate extramatrical stalk of *R. hyalothecae* Scherffel. The sporangia, however, of these two species differ from *R. pedicellatum* in their possession of apical teeth which are absent in the latter. Extramatrical, subsporangial stalks are also found in *Phlyctochytrium zygnematis* (Rosen) Schroeter and *P. dentatum* (Rosen) DeWildeman. A chytrid with a clavate subsporangial stalk which was believed to have a close affinity with *Rhizophydium digitatum* was figured by Sparrow (**1933**). The presence of this clavate extramatrical rhizoid is reminiscent of the characteristic main rhizoidal axis of species of *Rhizidium*. Indeed, sporangia of *Rhizophydium pedicellatum* have been observed which are not attached directly to the host but only by the stalk. This perhaps indicates that development occurs free in the medium, also a characteristic of *Rhizidium*. The stages in this *Rhizidium*-type of development were not, however, observed in *R. pedicellatum*. Since the sporangium is usually found in contact with the host and zoospore discharge is not by collective swarming it appears the affinities between this species and the genus *Rhizidium* are not pronounced. For these reasons this species is tentatively placed in the genus *Rhizophydium*.

ZYGORHIZIDIUM PLANKTONICUM Canter (FIG. 2, f–k)

This fungus, which is parasitic on *Synedra* sp., was found in Douglas Lake plankton during the last two weeks in August, 1954. The operculate sporangium is obpyriform and 8.5 to 13.5 μ long by 6 to 11 μ wide. The rhizoidal system is fairly extensive, branched, tapering, and arises from a main axis. From the sporangium are liberated 25 to 30 posteriorly uniflagellate zoospores (2 to 2.5 μ in diameter) which are spherical and contain a single eccentric refractive globule. Although observed only once, the resting spore was found to be sexually formed as a result of fusion between male and female thalli by a conjugation tube. This fungus is believed to be *Zygorhizidium planktonicum* Canter because

of its branched rhizoidal system, its zoospore size and its parasitism of the host, *Synedra*. The shape of the one resting spore observed resembles more the shape of *Zygorhizidium planktonicum* than that of the related species, *Zygorhizidium melosirae* Canter. However, the sporangium size falls more closely within the range of that described for *Z. melosirae* (7 to 14 μ high by 5 to 10 μ in diameter) than it does for that of *Z. planktonicum* (4 to 9 μ high by 3 to 8 μ broad). Furthermore, the rhizoids in *Z. melosirae* are unbranched or sparingly branched, the zoospores are 2.8 to 3.3 μ in diameter, and the host is *Melosira italica*. It may be possible that size differences between *Z. planktonicum* and the present organism are due to the fact that *Z. planktonicum* was described from preserved material.

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The author wishes to express his gratitude to Professor F. K. Sparrow for his criticism during the preparation of this paper and to Professor B. Meinecke of the Classical Studies Department, University of Michigan, for help with the Latin diagnoses.

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NEW SPECIES AND VARIETIES OF CLAVARIA FROM MICHIGAN

CHESTER R. LEATHERS[1]

(WITH 9 FIGURES)

During the summer and fall of 1951 and 1952, clavarioid fungi were collected in quantity throughout northern Michigan. Especially productive were the Tahquamenon Falls State Park near Paradise and the country surrounding the University of Michigan Biological Station near Cheboygan. In the course of studying collections obtained from these areas and those in the University of Michigan Herbarium, four new species and three new varieties were found.

A discussion of the distinguishing features of each taxon and a comparison of these with related species can be found in Leathers.[2]

The study was made under the direction of Dr. A. H. Smith, to whom I am indebted for his suggestions and criticism as well as for his help in securing collections and photographs. Sincere thanks are due to Prof. H. H. Bartlett for writing the Latin translations and to Dr. A. H. Stockard, Director of the University of Michigan Biological Station for making the facilities of the station available after the regular sessions had ended. I am also grateful to Dr. E. B. Mains and Dr. B. B. Kanouse for their generous loan of specimens from the University of Michigan Herbarium.

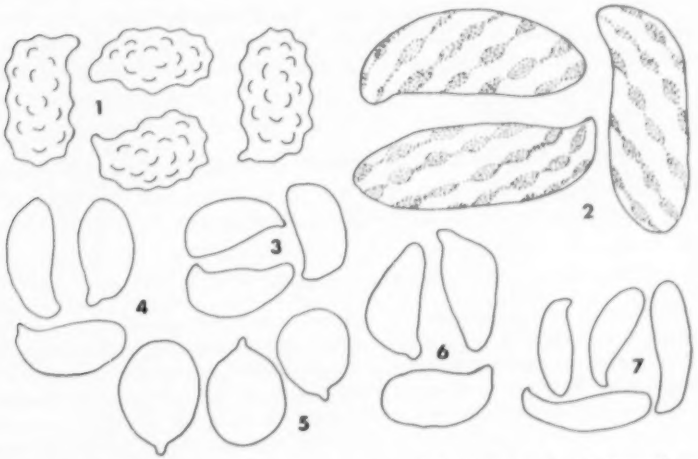
Clavaria cauliflorifformis sp. nov. FIGS. 1, 8.

Fructificatio 5-15 cm alta, 3-15 cm lata, ramosissima, compacta vel dense compacta aspectu varietati *Brassicae* cauliflori dictae similis; stipite albido vel brunneolo vetustate brunneo, brevi, basin versus angustato, laevi, aspectu variegato, maculis aliis aquatilibus aliis marmoreis, mycelio ut videtur nullo; hymenophoro ochraceo vel pallide brunneo vel atriore, interdum paulum et pallide rubescenti, apicibus aetate purpurascenter atrobrunneis; parte inferiore polychotoma superiore parte repetete dichotoma, ramis sursum parallelis et compressione mutua obtuse subangulosis, laevibus, infra ramificationes sensim crassioribus, primariis 3-12 mm

[1] Papers from the University of Michigan Herbarium, the Department of Botany and the University of Michigan Biological Station, University of Michigan, Ann Arbor, Michigan.

[2] The Genus *Clavaria* Fries in Michigan, doctoral dissertation, University of Michigan. 1955.

crassis, secondariis 2–5 mm crassis, quam primariis paulo longioribus, ultimis 1–2 mm crassis, apicibus obtusis vel raro subacutis; carni vel alba vel superficiei colori simili, in solutione sulfatis ferrici viridi, solida, sursum in ramis parenchymatosa, deorsum in stipite fragili partiatm aquatili et marmorea. Odor et sapor desunt. Sporae 8–10 μ longae, 4–5 μ crassae, massaliter ochraceae, verruculosae vel distincte verrucosae, ellipsoidales, 1–2-guttulatae, raro multiguttulatae, aureae in solutione chloralis hydrati et iodi, apiculo curvato. Basidia ex hymenio inseparabilia, circa 35–45 μ longa, 5–7 μ lata, clavata, sterigmatibus 2–4, 4–5 μ longis. Hymenium 90–100 μ crassum. Subhymenium laxe intertextum, hyphis ramossisimis hyalinis aspectu gelatinoso in solutione kalii hydrati. Trama ex hyphis laevibus parallelis vel subparallelis constans, cellulis cylindricis, modice longis, 3–9 μ diametro, infibulationibus numerosissimis et distinctis.



FIGS. 1–7. Spores of *Clavaria*. 1. *C. cauliflioriformis*. 2. *C. botrytis* var. *latispora*. 3. *C. fuscoferruginea*. 4. *C. rubicundula*. 5. *C. pulchra* var. *globulina*. 6. *C. luteo-tenerrima* var. *borealis*. 7. *C. phycophila*. (All about × 1875.)

Fruiting body 5–15 cm tall, 3–15 cm wide, much-branched, moderately compact to very compact with a cauliflower head-like appearance; stipe whitish to creamy brown, becoming "snuff brown" in age, very short, broad, 1–3 cm long and 1–3 cm wide, pointed at the base, smooth, with a watery-marbled appearance, arising directly from the substratum with no apparent mycelium; hymenophore uniformly creamy brown to light chocolate brown or darker ("pinkish buff" through "cinnamon buff" and finally to "snuff brown"), tips darkening in age to vinaceous brown ("pecan brown"), branching polychotomous below, dichotomous above, branches either moderately compact and erect and parallel with rounded angles between them, or with the branches tightly

compacted at the tips forming a cauliflower head-like growth, branches gradually enlarged upwards to the point of re-branching, smooth, primaries, 3–12 mm wide, soon re-branching, secondaries 2–5 mm wide, slightly longer than the primaries, tertiaries to 2 mm wide, slightly longer than secondaries, ultimates 1–2 mm wide, with a suggestion of further dichotomy, tips obtuse to rarely subacute; context white or colored as the surface, solid, pith-like in the branches and watery-marbled in the stipe, often brittle; taste and odor none. Spores 8–10 × 4–5 μ, pale ochraceous in mass, distinctly verruculose to verrucose, ellipsoid, 1–2 guttulate, rarely multiguttulate, with a curved apiculus, golden-yellow in Melzer's reagent; basidia approximately 35–45 × 5–7 μ, clavate, inseparable from the hymenium, with 2–4 sterigmata that are 4–5 μ long; hymenium 90–100 μ thick, subhymenium of loosely interwoven, much-branched hyaline hyphae that may appear sub-gelatinous in KOH; trama composed of hyaline, smooth and thin-walled, parallel to subparallel hyphae with cells that are nearly cylindric and moderately long, 3–9 μ wide, clamp connections very distinct and numerous.

Gregarious on sandy loam in a mixed growth of aspens and red oak near Topinabee, Mich., Sept. 1, 1951, coll. Smith and Leathers *319*, TYPE, in the University of Michigan Herbarium.

CLAVARIA BOTRYTIS var. **latispora** var. nov. FIG. 2.

Fructificatio inter species majores apparenter non distinguenda a var. *botryti*, ca. 8 cm alta, 7 cm lata, multiramosa omnino polychotoma; textura modice firma et fragili, partiatim aquata marmoreaque; stipite pallido, demum paululo rubescente, plus minusve distincto ex substrato oriente sine mycelio visibili; hymenophoro albido alutaceo, demum ochraceo vel brunneolo, saepe pallide rubritincto, apicibus obtusiusculis, rubentibus, siccitate ochraceibrunneis; ramis vel numerosis, brevibus, congestis, vel elongatis, erectis parallelis. Sporae plerumque 14–16 μ longae, 5.5–7 μ crassae, massaliter nondum observatae, a latere, obscure sigmoideae, a fronte et a tergo subellipticae, aspectu in sectione optica fere laeves, superficialiter oblique striatae cum excavationibus minutissimis linealiter ordinatis, ochraceae in solutione iodi et chloralis hydrati; membrana tenui. Basidia 55–75 μ longa, 9–11 μ lata, clavata, basi infibulata; sterigmatibus 4 erectis, ca. 7 μ longis. Hymenium ca. 90–100 μ crassum, a proliferatione cellularum prope basidiorum bases indeterminate accrescens. Subhymenium tenue ex hyphis laxis intertextis constans non aliter differentiatum. Trama ex hyphis hyalinis laevibus subparallelis constans, cellulis longis multiramosis cylindricis vel irregularibus, 4–9 μ crassis, ad septa haud constrictis, inter se distincte et abundanter infibulatis; membrana tenui.

Fruiting body appearing the same as in var. *botrytis*, about 8 cm tall and 7 cm wide, much-branched, large; stipe pallid, becoming pinkish in age, 1–2 cm long, 1–2 cm thick, more or less distinct, arising directly from the substratum without apparent mycelium; hymenophore whitish then alutaceous, tan or ochraceous with tints of pink, tips pale to deep

pink, drying ochraceous-brown, branching polychotomous throughout; branches very numerous, short and congested or elongate, erect, parallel, tips more or less obtuse; context white, unchanging, moderately brittle, watery-marbled, green in ferric sulphate; taste and odor not noted. Spores 14–16(–17) × 5.5–7 μ, deposit not noted, distinctly and diagonally pitted-striate, nearly smooth in optical section, in side view obscurely inequilateral, sub-elliptic in face view, thin-walled, 1-multiguttulate, ochraceous in Melzer's reagent; basidia 55–75 × 9–11 μ, clavate, clamped at the base, with 4 sterigmata that are about 7 μ long, straight and not diverging; hymenium ± 90–100 μ thick, suggestive of indeterminate growth; subhymenium thin, of loosely interwoven, hyaline, thin-walled hyphae, not otherwise differentiated; trama composed of hyaline, smooth and moderately thin-walled, subparallel hyphae with long, much-branched cells that are subcylindric to irregular in shape and 4–9 μ wide, not constricted at the septa; clamp connections distinct and numerous.

Single, on humus in open oak woods near Ann Arbor, Mich., coll. A. H. Smith, Sept. 27, 1940, under the name of *Clavaria rufescens* (TYPE, in the University of Michigan Herbarium).

Clavaria fuscoferruginea sp. nov. FIG. 3.

Fructificatio 3–6 cm alta, 2–7 mm crassa, simplex, cylindrica vel clavata, saepe fusiformis et compressa, ex substrato oriens absque mycelio obvio; stipite vivo indistincto, ca. 5 mm longa, 2 mm crassa, sed siccato distincto et luteo; hymenophoro curvato, raro recto, unicolore fusco-ferrugineo, siccitate atriore, laevi vel ruguloso etiamque bilateraliter profunde sulcato, solido in exemplis cylindricis, tubuloso in exemplis grandioribus compressis, fragilissimo, sapore odoreque simili seminibus noviter effossis *Arachis hypogaeae*, vel solum in speciminibus recentibus. Sporae 5.5–7 μ longae, 3–3.5 μ diametro, laeves, oblonge ellipsoidales vel late ellipsoidales, prope apiculum ampliatae, eguttulatae vel 1-guttulatae, minute apiculatae, in solutione chloralis hydrati cum iodo hyalinae, membrana tenui. Basidia 33–45 μ longa, 6–7 μ lata, breviter clavata, pigmento pallide brunneo plena, sterigmatibus plerumque 4, raro 2, late divergentibus, vel rectis vel curvatis. Hymenium 55–65 μ crassum. Subhymenium crassiusculum plus minusve prosenchymatosum. Trama ex hyphis hyalinis vel brunneis, laevibus, compactis, parallelis et cullulis brevibus inflatis vel cylindricis, 7–18 μ latis, 20–110 μ longis constans, absque fibulationibus visibilibus.

Fruiting body 3–6 cm tall, 2–7 mm wide, simple, cylindric or clavate, often fusiform and laterally compressed; stipe poorly defined, ± 5 mm long and 2 mm wide, distinct as a yellowish colored zone when dried, arising directly from the substratum with no apparent mycelium; hymenophore uniformly dark brown ("snuff brown" to "cinnamon brown") or darker, drying to blackish-brown, smooth or longitudinally rugulose with a deep groove on each side, curving, rarely straight, tip obtuse; context pale brown or "snuff brown," black when dried, solid in cylindric specimens, hollow in the larger compressed ones, very brittle, friable

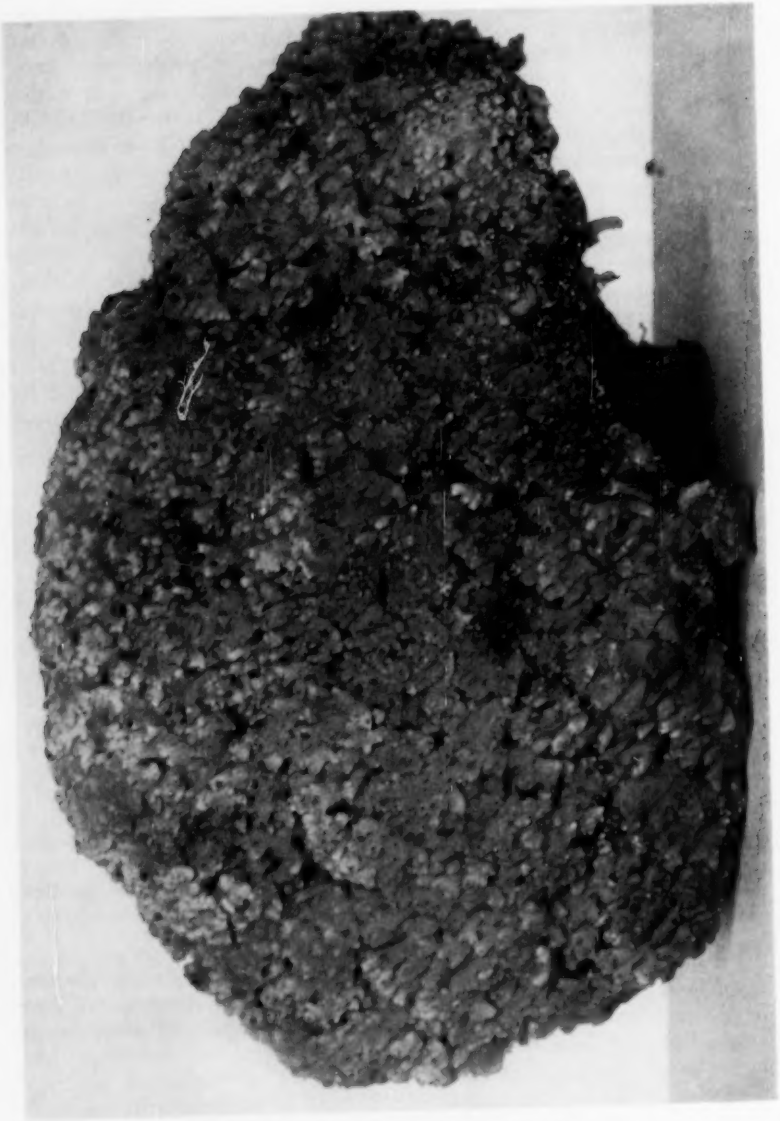


FIG. 8. *Clavaria cauliflioriformis*, × ¾.

when sectioned; taste and odor strong, of unripe peanuts or rarely lacking, not distinctive when dried. Spores 5.5–7(–8) × 3–3.5(–4) μ, smooth and thin-walled, oblong-ellipsoid to broadly ellipsoid, wider near the apiculate end, aguttulate or rarely 1-guttulate, minutely apiculate, hyaline in Melzer's reagent; basidia 33–45 × (4–)6–7 μ, short, clavate, filled with a brownish pigment, with (2–)4 sterigmata that are widely divergent and straight or curved; hymenium 55–65 μ thick, subhymenium somewhat prosenchymatous, rather broad; trama composed of hyaline or brown-pigmented smooth and thin-walled hyphae that are compact, parallel and with short, inflated, cylindrical cells, (3–)7–18 μ wide and mostly 20–110 μ long; clamp connections not present so far as could be determined.

Gregarious, sometimes caespitose on humus in deciduous forests at Pellston hills hardwoods near Pellston, Mich., Aug. 11, 1951, coll. Smith (Leathers *268*), TYPE, in University of Michigan Herbarium.

Clavaria rubicundula sp. nov. FIGS. 4, 9.

Fructificatio 4–11 cm alta, 2–7 mm crassa, simplex vel raro bifurcata, cylindrica vel anguste clavata, interdum paululo compressa; stipite indistincto, colore pallide eburneolo, 1–1.5 cm longo, tereti, laevi, ex substrato sine mycelio obvio oriente; hymenophoro unicolori, pallide ochraceo, apicibus brunescentibus, obtusis vel subacutis, raro bifurcatis; carni pallide ochracea, fragili, odore saporeque iodo diluto simili. Sporae plerumque 5.5–8.5 μ longae, 3–4 μ latae, laeves, ellipsoidales vel oblonge ellipsoidales, vix apiculatae, in solutione chloralis hydrati cum iodo hyalinae, membrana tenui. Basidia 35–50 μ longa, 5.5–7.5 μ crassa, clavata, sterigmatibus (2–)4, sine infibulationibus. Hymenium 35–50 μ crassum. Subhymenium ca. 25 μ crassum. Trama ex hyphis parallelis constans, cellulis brevibus cylindricis vel subinflatis sed ad septa constrictis, 25–70 μ longis, 7–13 μ crassis, sine infibulationibus.

Fruiting body 4–11 cm tall, 2–7 mm wide, simple or rarely bifurcate, cylindric or narrowly clavate or slightly compressed laterally; stipe poorly defined as a pale creamy buff (pale "vinaceous buff") zone 1–1.5 cm long and 1.5–2 mm wide, terete, smooth, arising from the substratum usually without apparent mycelium; hymenophore uniformly creamy buff (near "vinaceous buff" or "vinaceous pink"), tips becoming brownish, tip obtuse to subacute, rarely bifurcate; context "vinaceous buff" or "vinaceous pink," very brittle, friable when sectioned; taste none, odor suggestive of tincture of iodine. Spores (5–)5.5–8.5 × 3–4 μ, smooth and thin-walled, ellipsoid to oblong-ellipsoid, faintly apiculate, hyaline in Melzer's reagent; basidia 35–50 × 5.5–7.5(–9) μ, clavate, not clamped at the base, with (2–)4 sterigmata; hymenium 35–50 μ thick; subhymenium about 25 μ wide; trama of hyaline, parallel hyphae with short, inflated cells that are usually cylindric or barrel-shaped and 7–13(–19) μ wide, 25–70 μ long; clamp connections not present so far as could be determined.

FIG. 9. *Clavaria rubicundula*, ×1. (Photograph by A. H. Smith.)

Densely caespitose and fasciculate on sandy loam and humus in Pellston hills hardwoods near Pellston, Mich., throughout July and August, 1951 and 1952, Smith *32898*, TYPE, in the University of Michigan Herbarium.

CLAVARIA PULCHRA var. **globulina** var. nov. FIG. 5.

Fructificatio simplex, 1.5–3.5 cm alta, 1–2 mm lata, cylindrica, erecta, stricta vel varie curvata; stipite indistincto vel raro distincto colore albido ex substrato orto sine mycelio visibili; hymenophoro unicolori saturate aurantiaco, cylindrico, recto vel curvato, apicem versus gradatim attenuato, apice acuto in exemplis minoribus sed interdum obtuso in grandioribus; carni pallide flava vel pallide aurantiaca, solida, textura modice fragili, cerosa nec lenta. Sapor nullus. Odor nullus vel indeterminabilis. Sporae 5.5–7 μ longae, 5.5–6.5 μ crassae, laeves, globosae vel subglobosae, uniguttulatae, hyalinae in solutione chloralis hydrati cum iodo, membrana tenui, apiculo 0.5–1 μ longo. Basidia plerumque 25–40 μ longa, 6.5–7.5 μ lata, saepissime breviter clavata, saepe prope mediam abrupe angularia, basi fibulosa, sterigmatibus 4, 5–9 μ longis. Hymenium 25–30 μ crassum. Subhymenium angustissimum ex hyphis multiramosis et aurantiacis textum. Trama ex hyphis pallide aurantiacis laevibus compactis parallelis constans, cellulis brevibus vel longis, cylindricis, 3–6.5 μ latis; infibulationibus numerosis, parvis, inconspicuis.

Fruiting body 1.5–3.5 cm tall, 1–2 mm wide, simple, cylindric, erect, straight or somewhat curved; stipe indistinct or rarely distinct as a translucent whitish zone at the base, straight, arising directly from the substratum with no apparent mycelium; hymenophore uniformly deep orange ("capucine orange"), cylindric, straight or somewhat curved, gradually attenuated in the apical region, tip blunt, often acute in smaller specimens; context pale yellow to pale orange, solid, waxy, not very brittle but not tough; taste none; odor faint or none. Spores 5.5–7 $\times$ 5.5–6.5 μ, smooth, thin-walled, globose to subglobose, 1-guttulate with the apiculus 0.5–1 μ long, hyaline in Melzer's reagent; basidia 25–40(–50) $\times$ 6.5–7.5 μ, mostly short, clavate, often sharply bent, clamped at the base, with 4 sterigmata that are 5–9 μ long; hymenium 25–30 μ thick; subhymenium very narrow, of orange-pigmented, much-branched hyphae; trama composed of pale orange-pigmented, smooth and thin-walled, compact, parallel hyphae with short to long, cylindrical cells that are 3–6.5 μ wide; clamp connections numerous, small and inconspicuous.

Caespitose on sandy loam in Pellston hills hardwoods near Pellston, Mich., July 21, 1952, coll. A. H. Smith (Leathers *497*), TYPE, in the University of Michigan Herbarium.

CLAVARIA LUTEO-TENERRIMA var. **borealis** var. nov. FIG. 6.

Fructificatio 2–5 cm alta, 1.5–6 mm crassa, simplex, ex humo vel solo humoso arenoso oriens sine mycelio obvio; stipite indistincto sed luteolo et subbulboso, 2–4 mm longo, 3–5 mm crasso, siccitate a hymenophoro coloratione vines-

centi vel rubribrunneo distincto; hymenophoro luteo citrino, siccitate pallide vel saturate ochraceo, tereti vel fusiformi, lateraliter subcompresso et canaliculato, recto vel sensim curvato, gradiatim apicem versus angustato, apice rotundato vel subacuto; textura cum superficie concolori solida, fragilissima. Sapor nullus. Odor acris, tincturam iodi reminiscens. Sporae 6–9 μ longae, 3–4 μ latae, laeves, tenuiter circumvallatae, ellipsoideae vel oblongae ellipsoideae, saepe prope basim apiculatam sensim expansae, hyalinae in solutione Melzeri, guttulis 0–1, apiculo 1–2 μ longo. Basidia 30–35 μ longa, 6.5–9 μ crassa, breviter clavata, sterigmatibus 2–4, 2–4.5 μ longis, vel incurvis vel rectis lateque divergentibus. Hymenium 25–60 μ crassum apparenter indeterminate crescens. Subhymenium indistinctum. Trama ex hyphis luteis laevibus tenuiter vallatis parallelis constans, cellulis plerumque 9–11 μ latis, 20–40 μ longis, breviusculis inflatis cylindricis sed ad septa distincte constrictis, granulorum pigmentosorum luteorum plenis; infibulationibus rarissimis sed cellulis haud infrequenter ramo abortivo prope mediam partem praeditis.

Fruiting body 2–5 cm tall, 1.5–6 mm wide, simple; stipe poorly defined as a pale yellow bulbous portion at the base, 2–4 mm long and 3–5 mm thick, distinct from the hymenophore when dried as a vinaceous or maroon colored zone, arising directly from the substratum with no apparent mycelium; hymenophore uniformly lemon yellow ("citron yellow"), drying "chamois" to "ochraceous," terete or somewhat fusiform and somewhat laterally compressed and grooved on the sides, straight or slightly curved, slightly and gradually attenuated toward the apex, tip rounded or subacute; context concolorous with the surface, solid, very brittle, friable when sectioned; taste none; odor sharp, reminding one of tincture of iodine. Spores 6–9 × 3–4 μ, smooth, thin-walled, ellipsoid to oblong-ellipsoid, often wider near the apiculate end, aguttulate or rarely 1-guttulate, the apiculus 1–2 μ long, hyaline in Melzer's reagent; basidia 30–35 × 6.5–9 μ, short, clavate, with 2–4 sterigmata that are 2–4.5 μ long with many incurved, the rest straight and somewhat divergent; hymenium 25–60 μ thick, apparently of indeterminate growth; subhymenium not distinctly differentiated; trama composed of yellow-pigmented, smooth and thin-walled, compact, parallel hyphae with moderately short, inflated cells that are cylindrical and distinctly constricted at the septa, filled with a yellowish pigment in the form of granules and (4.5–)9–11(–16) μ wide, 20–40 μ long; clamp connections extremely rare but the cells infrequently with an abortive branch in the middle portion of the wall.

Gregarious, sometimes caespitose on sandy loam and humus in Pellston hills hardwoods near Pellston, Mich., July 21 and 22, 1952, coll. A. H. Smith and C. R. Leathers (Leathers *540*), TYPE, in the University of Michigan Herbarium.

Clavaria phycophila sp. nov. FIG. 7.

Fructificatio 8–12 mm alta, 1–2 mm crassa, plerumque simplex, raro prope apicem bifurcata, clavate, raro cylindrica, phycophila, sine odore vel sapore; stipite

indistincto translucenti albido 2–3 mm longo, 1–2 mm crasso, tereti, sursum sensim crassiore, laevi, ex substrato elevato sine mycelio obvio; hymenophoro translucenti colore intus et extus ochraceo-aurantiaco vel pallidiore, siccitate aurantiaco, apice plerumque obtuso albidulo fere transparenti. Sporae plerumque 6.5–10 μ longae, 2–3.5 μ latae, hyalinae, laeves, membrana tenui, allantoideae vel anguste fusiformes, hyalinae in solutione chloralis hydrati et iodi, intus granuliferae vel 1–3 guttulatae, apiculo obscuro vel nullo. Basidia parva, 15–30 μ longa, 4–7 μ lata, breviter clavata, sterigmatibus apparenter 4. Hymenium 15–35 μ crassum, cystidiis numerosis vel clavatis vel ampulliformibus vel raro ventricoso-fusiformibus, profunde inclusis vel exsertis, plerumque 20–40 μ longis, 7 μ latis. Subhymenium angustum. Trama ex hyphis intertextis vel irregulariter paene parallelis constans, cellulis irregularibus, inflatis 4.5–13 μ diametientibus, infibulationibus in infructificationibus aliis distinctis aliis indistinctis.

Fruiting body 8–12 × 1–2 mm, usually simple, rarely bifurcate at the apex, clavate, rarely cylindric, phycophilous; stipe poorly defined as a basal translucent-whitish zone 2–3 mm long and 1–2 mm wide, terete, slightly enlarging upwards, smooth, arising directly from the substratum with no apparent mycelium; hymenophore translucent "ochraceous orange" or paler, drying to "apricot orange" with a translucent tip that is usually obtuse or rarely bifurcate; context "ochraceous orange" or paler; taste and odor none. Spores (6–)6.6–10 × 2–3.5(–4) μ, hyaline, smooth and thin-walled, allantoid to narrowly fusiform, with granular contents or 1–3 guttulate, apiculus not noticeable, hyaline in Melzer's reagent; basidia 15–30 × 4–7 μ, small, short-clavate, apparently with 4 sterigmata; hymenium 15–35 μ thick, with numerous clavate or flask-shaped or rarely fusoid-ventricose cystidia deeply embedded or often with their necks projecting and mostly 20–40 × 7 μ; subhymenium narrow; trama composed of hyaline, smooth and thin-walled, interwoven to irregularly sub-parallel hyphae with irregular, inflated cells (3–)4.5–13 μ in diameter; clamp connections distinct in some fructifications but not in others.

Gregarious on sandy and clay loam that is covered with *Chroococcus* and *Mougeotiopsis* in Tahquamenon Falls State Park near Paradise, Mich., July and August, colls. Smith and Leathers (Leathers *465*), TYPE, in the University of Michigan Herbarium.

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THE CYTOLOGY OF XENOGLOEA ERIOPHORI [1]

C. J. KAO [2, 3]

(WITH 40 FIGURES)

The fungus discussed in this paper was first described by Bresadola in 1891 (**2**) as *Kriegeria Eriophori*, from pustules occurring on the leaves of *Eriophorum angustifolium* in Saxony. Bresadola assigned the genus to the Tuberculariaceae. Saccardo, in a letter to Bresadola in 1890, named it as *Septogloeum? dimorphum* and published it under this name in 1892 (**15**) in the Sylloge, including it in the Melanconiaceae. Höhnel (**7**) restudied the species and stated that it was closely related to *Platygloea* and tentatively renamed it *Platygloea Eriophori* (Bres.) Höhnel, placing it in the Auriculariaceae. H. and P. Sydow (**18**) erected for it a new genus *Xenogloea* in the Auriculariaceae, changing the name to *X. Eriophori*.

Xenogloea is widely distributed in Europe. It was first reported from North America by J. J. Davis (**3**) in Wisconsin. The description given by Martin (**10**) was based on five collections by J. J. Davis and two collections by H. C. Greene, all on leaves of *Scirpus atrovirens* in Wisconsin, as well as on European collections. It is now known to occur in Iowa on the same host.

Xenogloea is parasitic on leaves of *Scirpus* and *Eriophorum*. The present study is based on two collections occurring on *Scirpus*. It has elongated, pustular fructifications which are usually situated between major veins on the abaxial surface of the leaves, causing yellow discoloration, then brown necrotic areas. In dry specimens, the pustules are coalescent, horny and yellow. Soft, gelatinous, gray pustular masses can be seen when the specimen is moistened. These pustular masses are composed of early and late stages of basidia and germinating spores.

[1] Portion of a thesis Part I submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the State University of Iowa, June, 1955.

[2] The writer wishes to express her gratitude to Professor G. W. Martin for his guidance and encouragement during the course of this study. She also wishes to acknowledge the Nancy Skinner Clark Fellowship for 1952–53 from Vassar College and the Frank M. Shu Scientific Fellowship for 1954–55 from the China Institute in America, without which this work could not have been done.

[3] Mrs. Stephen S. Wang, Jewish Hospital Clinical Laboratory, Cincinnati 29, Ohio.

MATERIALS AND METHODS

The materials used for this study were collected by H. C. Greene on the leaves of *Scirpus atrovirens* in Wisconsin in July, 1951, and by R. J. Bandoni and J. H. B. Garner on the same host in Iowa City in July, 1954.

Greene's specimens arrived at Iowa City packed with dry ice and were in good condition. Immediately upon receipt, the material was fixed in FPA solution.

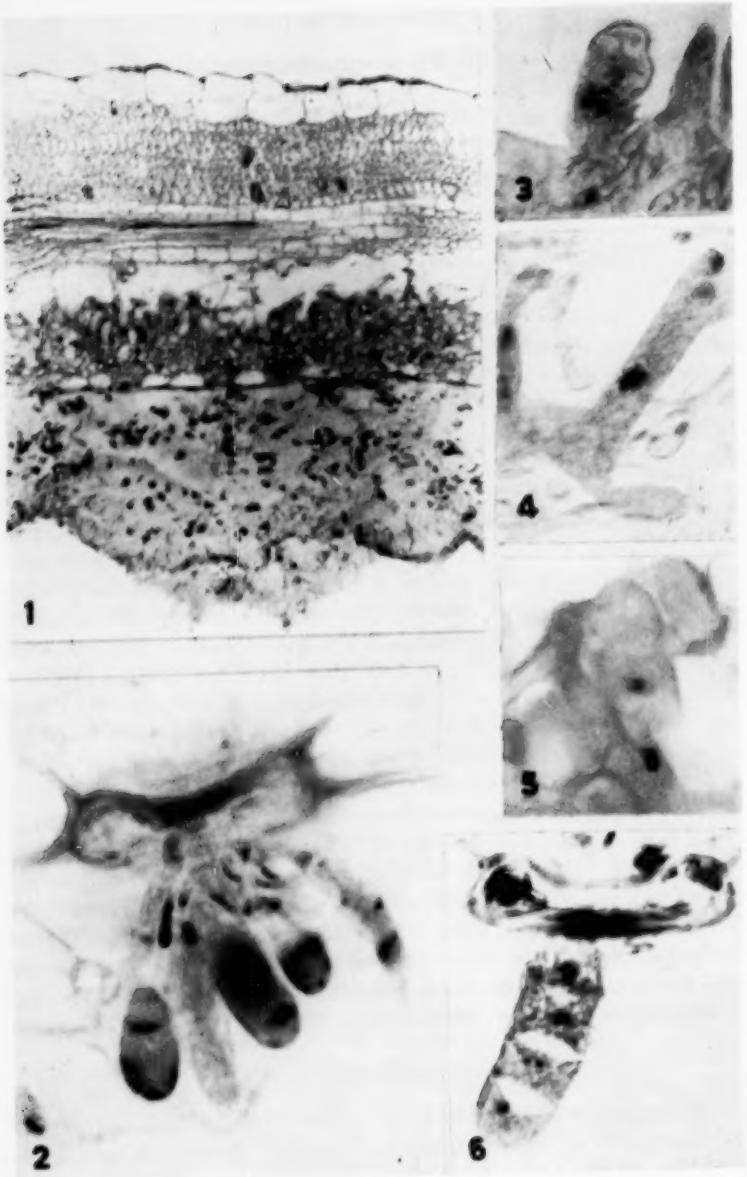
The Bandoni and Garner specimens (G. & B. *54–16*) were fixed with FAA and Navashin's solutions.

A part of each of these materials was dehydrated in a butyl alcohol series and was then imbedded in paraffin and sectioned parallel with the major veins of the leaf at 8 μ. In order to study the probasidia and hyphae inside the leaves, some materials were sectioned at 12 and 15 μ.

Heidenhain's Iron-alum Hematoxylin stain (**8**) with Orange G or Fast Green as a counter-stain proved to be the most satisfactory staining method for bringing out the nuclear details and was employed for all the slides used in the present study. Several smear methods, such as Aceto-carmine, Propio-carmine, Aceto-orcein and Trypan Blue, were tried. The Trypan Blue method of Hoffmeister (**6**) proved to give the best results, hence it was used for the cytological investigation of basidia and basidiospores in the pustules on the leaf surfaces. Small amounts of material were transferred directly from the FAA solution or Navashin's solution to a drop of Trypan Blue on a slide and allowed to stand for about 30 seconds. A cover slip was applied to each drop and pressed down until the material was spread out. By this method, the nuclei were stained bright red and the cytoplasm pinkish blue at first. As the stain penetrated more and more into the cells, the nuclei gradually changed from bright red to deep purplish blue and the cytoplasm changed from pinkish blue to light blue. No counter stain was needed if a proper amount of Trypan Blue was applied. Although Trypan Blue can be used for staining nuclei, it is inadequate for differentiating chromatin materials in the nuclei. Since it was very effective in staining nuclei and extremely simple in preparation, it was used in this study to supplement the more precise stains.

OBSERVATIONS AND RESULTS

In the infected leaf tissues, the fungal hyphae branch in the intercellular spaces of the outer layers of spongy parenchyma cells of the leaf beneath the lower epidermis. The inner layer of spongy paren-



FIGS. 1-6.

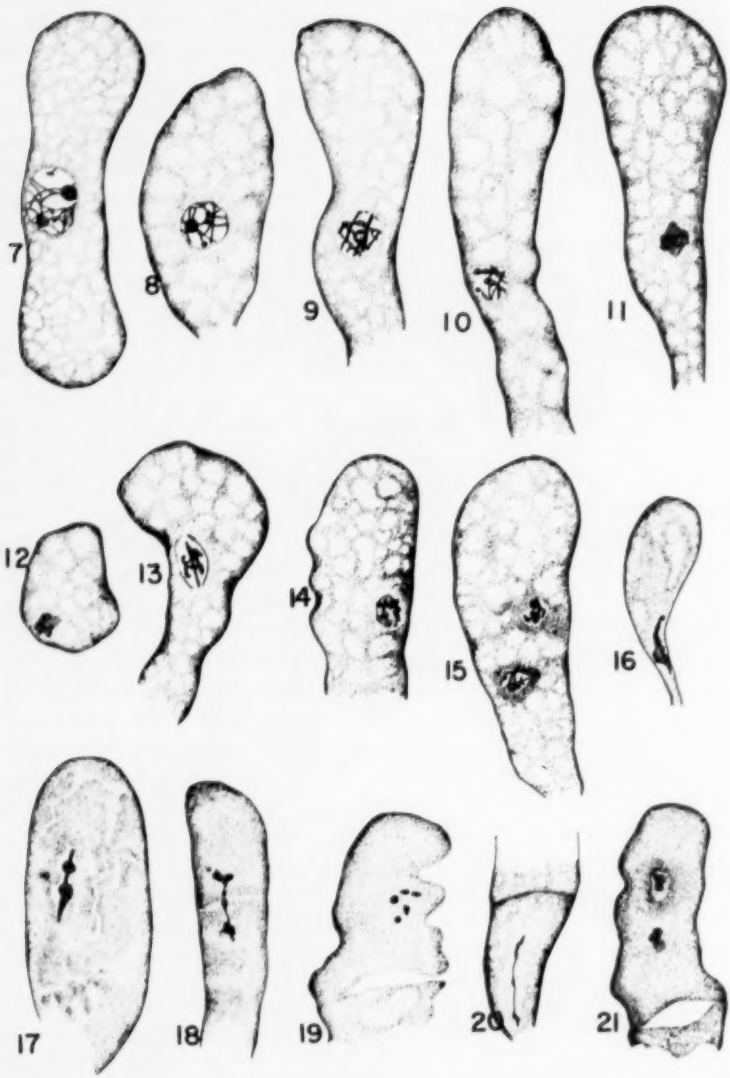
chyma and the veins are usually distorted by the masses of compact hyphae as well as by probasidia which replace the inner spongy layers and veins (FIG. 1). Most of the hyphae inside the leaf gather from all sides toward the air chambers under the stomata and form a dense hyphal cluster beneath each stoma. A few hyphae pass through each stoma and spread radially outward. In a paradermal section, these hyphae may be seen to meet and form a net-like coat scattered on the lower surface of the leaf. The young basidia are usually formed immediately as the hyphae emerge from the stoma. Thus the masses of basidia and germinating spores, imbedded at random in gelatinous material, form pustules on the surface of the lower epidermis of the leaf. There is no evidence to show that the hyphae or young basidia in the leaf burst through the epidermis and form pustules. In all cases observed, stomata provide the passage-way for the probasidial hyphae or young basidia (FIGS. 2, 6).

In the compacted fungus hyphae inside the leaf, in Heidenhain's Hematoxylin stained slides, the nuclei may be seen as deep purple dots. They occur in pairs. Clamp-connections are abundant inside the leaf, a feature which is unusual in parasitic members of this group. Numerous probasidia are also found inside the leaf. They are cylindrical and occasionally branch (FIGS. 4, 7–15). They vary from 25 to 45 μ in length and 7 to 12 μ in width. Two haploid nuclei migrate toward the center of the probasidium and come in contact. In FIG. 3 nuclear membranes as well as nucleoli may be observed clearly in the probasidium.

The membranes of the two haploid nuclei in the probasidium disappear in the area of contact. These two haploid nuclei then fuse to form a large diploid nucleus (6.5 μ). The fused nucleus increases in size and is larger than the sum of the two haploid nuclei. The fused nucleus in FIG. 7 is in the very early stage of prophase; the chromatin materials of the two nuclei are still distinguishable. Two nucleoli and two sets of chromatin material can be seen in the drawing.

It is difficult to find out when synapsis begins in the diploid nucleus. However, FIG. 8 seems to show chromosomes at zygotene stage. The chromosomes are long and slender, and are arranged in a net-like

FIGS. 1–6. 1. Compact hyphae and probasidia replace the inner spongy layers and veins of the leaf. The masses of basidia and germinating spores form pustules on the surface of the lower epidermis of the leaf, × 100. 2. Saccate structure and young basidia at the stoma on the lower leaf surface, × 914. 3. Two haploid nuclei attached closely to each other in the probasidium, × 914. 4. Probasidium, inside leaf, × 914. 5. "Rod-shaped spindle" in the probasidium, × 914. 6. Mature 4-celled basidium at the stoma, × 914.



FIGS. 7–21.

fashion. Nucleoli may be seen distinctly in these figures. The nuclear membranes can be distinguished at this stage. About 80% of the nuclei in the probasidia are found in zygotene stage.

FIG. 9 appears to show the chromosomes in the pachytene stage. Although the chromosomes are too narrow to show the formation of chromatids, yet their thicker and shorter appearance suggests the possibility of this stage. The nucleolus still remains in the nucleus.

FIG. 10 is a late diplotene stage, where the double nature of the chromosomes may be seen. The chromosomes are much shorter at this stage and begin to separate. The presence of chiasmata among the chromosomes can be observed. The nucleoli have disappeared. The size of the nucleus is much smaller than in early stages (3.5 μ in diameter). In fact, nuclei gradually become smaller after the early prophase. Nuclear membranes cannot be distinguished.

The diplotene stage is followed by diakinesis. However, none of the preparations gave a typical figure of diakinesis.

Metaphase chromosomes and plates are shown in FIG. 11, in which two pairs of chromosomes have begun to move toward the poles. The spindle fibers are too fine to be distinguished in such small structures, but the region in which they would be expected to occur is densely stained. The nuclear membrane has completely disappeared. FIG. 12 shows one cross section of the probasidium with metaphase plate. The polar view of the metaphase plate is shown as a darkly stained area with dot-like chromosomes. The chromosome number of *Xenogloea* at metaphase I appears to be eight.

In FIG. 14, the homologous chromosomes separate and pass to the opposite poles; this is anaphase.

Among hundreds of probasidia examined during this study, 16 cases have been found possessing the features shown in FIGS. 5 and 13. The appearance of the chromosomes suggests late metaphase or early anaphase, where homologous chromosomes move toward the poles. However, the straight dark lines remain unexplained, as does their direction. The direction of some of the dark lines is not exactly parallel with the

FIGS. 7–21. Made with the aid of camera lucida from Heidenhain's Hematoxylin preparations, all × 1500. 7. Newly fused diploid nucleus in the probasidium. 8. Prophase I, zygotene stage. 9. Prophase I, pachytene stage. 10. Prophase I, late diplotene stage showing the separating chromosomes and chiasmata. 11. Metaphase I. 12. Metaphase I, polar view. 13. "Rod-shaped spindle" in the probasidium. 14. Anaphase I. 15. Telophase I. 16. Chromatin material migrating through the hypha into the young basidium in the pustule on the leaf. 17. Anaphase I. 18. Early telophase I. 19. Prophase II. 20. Anaphase II. 21. Telophase II.

axes of the probasidia. They are stained deep purple, like the chromosomes, by Heidenhain's Hematoxylin. All these sixteen probasidia were found inside a leaf which had been fixed with Navashin's solution. The dark lines appear to be similar to those observed by Guilliermond (**5**) in *Schizosaccharomyces octosporus* and by Pady (**14**) in *Hyalopsora aspidiotus*. Pady called this structure a rod-shaped spindle.

Telophase can be observed in FIG. 15; the two sets of chromosomes are well separated and surrounded by dense cytoplasm. The resultant two nuclei are soon isolated by a transverse wall. The second meiotic division probably takes place in the 2-celled basidium in the leaf and a mature, transversely 3-septate basidium is then formed. In the present study, only about five 4-celled basidia have been seen within the leaf. Occasionally, basidiospores and germinated or budded basidiospores can be observed within the leaf. However, numerous mature 3-septate basidia are found at the stomata or in the pustules on the leaf surfaces. Therefore, all the observations on the second division in meiosis and the formation of basidiospores, as well as on the development of basidiospores, were made from the basidia and basidiospores in the pustules on the lower surface of the leaf.

FIG. 17 shows the chromosomes clustered into two groups, each arranged along the spindle toward the pole. This is at anaphase I in the probasidium in the pustule on the leaf. In FIG. 17, a deeply stained dot can be found beside one set of the chromatin material. This is probably the nucleolus, which had not disappeared. FIG. 18 is at early telophase where the "spindle" is still visible. Most of the chromatin materials had already accumulated at each spindle pole. Two nuclei are then formed in the basidium. The resultant two nuclei are soon isolated by a transverse wall as shown in FIG. 29. The second division takes place immediately in the 2-celled basidium. FIG. 19 shows the chromosomes in late prophase II. Four chromosomes and one nucleolus are visible. FIG. 20 is at late anaphase II. FIG. 21 is in telophase II. As the second meiotic division is completed, a mature, transversely 3-septate basidium is formed (45–60 $\mu \times$ 10–15 μ) (FIGS. 6, 31). The basidia are always stalked. The second divisions of these two nuclei may not be simultaneous, as shown in FIG. 30. In some cases, in the FPA fixed material, the basidial cells appear to have their walls separated from one another by a space (FIG. 6). It is inferred that this is due to plasmolysis, but Sanwal (**16**), as noted below, has a different explanation.

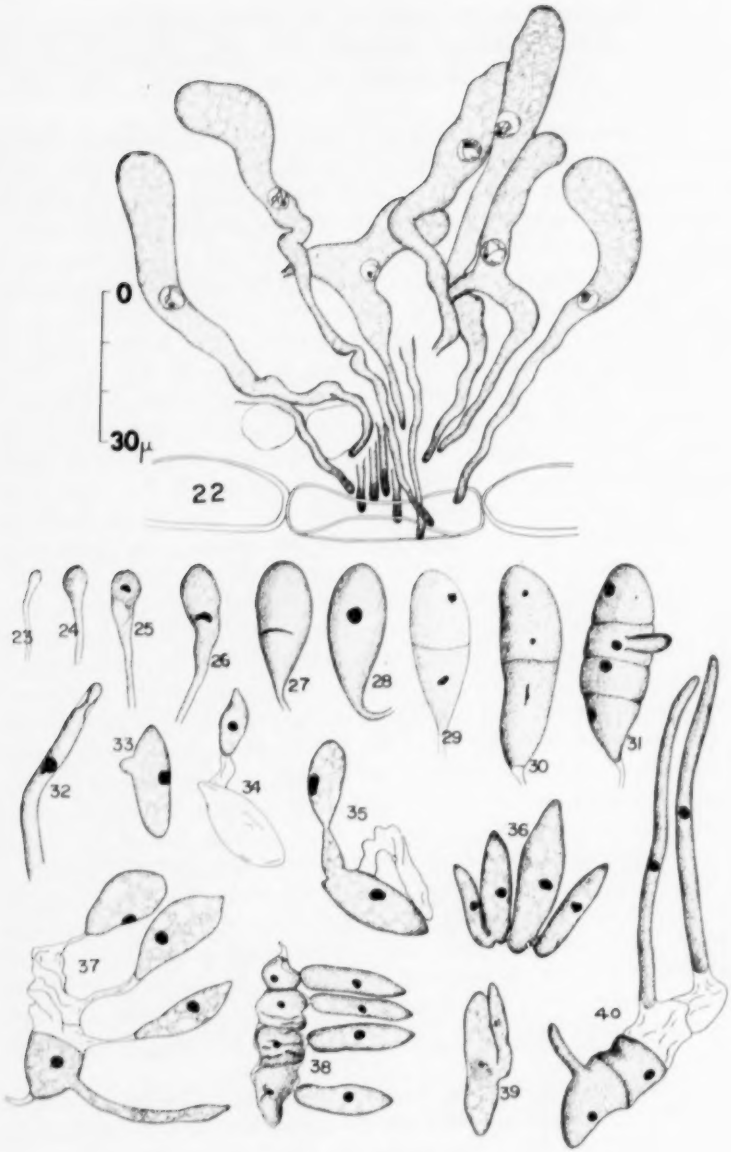
The above observations raise the question concerning the relationship between those probasidia in the leaf and those on the surface of the leaf.

By carefully examining the slides, it is found that at the bases or center of the probasidia, hyphae are developed (FIG. 22). They grow toward the stoma. The tips of the hyphae are densely stained. They appear to pass through the stoma.

The most striking appearance is that clusters of young or mature basidia and hyphae are frequently found at the stomatal opening. This is borne out by examination of both the sectioned materials and the smear-stained materials. Among the hyphae passing through the stomata, some are relatively young hyphae which are stained deep purple with Heidenhain's Hematoxylin or purplish red with Flemming's triple stain and some are old hyphae which do not take these stains at all. Frequently at the tips of such darkly stained young hyphae, there are saccate structures, such as those shown in FIGS. 23 and 24. These structures, like the hyphae, take a deep purple color with Hematoxylin and deep red with Flemming's triple stain and pink to red with Trypan Blue. They vary in size; the smallest ones are about 2.5 μ in width. No nuclei can be seen in the smaller structures. In the larger ones, only one nucleus can be found (FIGS. 25–28). Most of the nuclei are crescent-shaped (FIGS. 26, 27); some are oblong. In some cases, below the crescent-shaped nucleus is a vacuole-like structure (FIG. 25).

The shape of these saccate structures suggests young basidia, since all the intermediate stages between the smallest saccate structure and the mature basidia are found. It is suggested that the development of the basidia in *Xenogloea* is as follows: Karyogamy occurs in the probasidium in the leaf and is followed by meiosis. Meiosis may be completed in the probasidium inside the leaf, forming the 3-septate mature basidium, or may be completed much later. At the bases of the probasidia, hyphae are developed and grow toward the stoma. They appear to pass through the stoma. It is possible that at the tips of these hyphae the saccate structures are formed and gradually increase in size. In the meantime, diploid nuclei in the probasidia migrate through the hyphae and enter into the saccate structures, in which meiosis is completed. The crescent-shaped nuclei in the small saccate structures are probably due to the movement of the nuclei as well as the growth of the young basidia. FIG. 16 shows a nucleus migrating through the hypha into the saccate structure. This is additional evidence to support the above hypothesis.

Usually each cell of the basidium produces a slender epibasidium and then the nucleus migrates into the epibasidium and the cell becomes empty (FIGS. 31, 40). Sterigmata are formed at the tips of these epibasidia and basidiospores are produced (FIGS. 32, 37). The nucleus



FIGS. 22–40.

in each epibasidium moves into the basidiospore, which is then released. The lengths of the epibasidia are not the same; some are as short as 5 μ, others are up to 100 μ. Sometimes the cell in a basidium produces a sterigma and basidiospore directly. The nucleus in the cell divides to form 2 nuclei and one migrates into the basidiospore. In most cases the nucleus and the cytoplasm in this cell become shriveled (FIG. 38).

The basidiospores are ovate to subfusiform and uninucleate (18–30 $\times$ 7–11 μ, FIGS. 33–39). Basidiospores germinate by repetition, as shown in FIGS. 33–35. The basidiospore produces a germ tube and at its tip, a similar spore is formed. The nucleus and the cytoplasm move into the newly formed spore and the original one shrivels. Sometimes, the nucleus divides and one of the daughter nuclei moves into the secondary spore. Sometimes, the secondary spore germinates by repetition also (FIG. 35). Basidiospores also may germinate either by budding off elongate or oval cells (FIG. 39) or by producing one to four sessile secondary spores arranged in fan-like fashion (FIG. 36). The nucleus in the spore divides and migrates into the secondary spores or buds.

DISCUSSION

It is of interest to note that in *Xenogloea*, clamp-connections and probasidia are found abundantly on the hyphae inside the leaf instead of in the pustules on the leaf surface. The fungus has adjusted itself to the host environment and develops within the leaf. This suggests that *Xenogloea* is a highly specialized parasitic fungus among the Auriculariaceae. The effect on this fungus when its host, *Scirpus*, becomes withered and the manner of infection are as yet unknown.

Stages in meiosis in the probasidia of *Xenogloea* can be observed.

FIGS. 22–40. Made with the aid of camera lucida. $\times$ 750. Figs. 22, 39 from Heidenhain's Hematoxylin preparations. Figs. 23–28, 40 from Trypan Blue preparations. 22. Hyphae grown from the basal or central portion of the probasidium inside the leaf toward the stoma. 23, 24. Very young saccate structures at the stoma. 25, 26, 27. Larger saccate structures with crescent-shaped or oblong nuclei. 28. Large saccate structure or young basidium with normal nucleus. 29. Two-celled young basidium. 30. Division of the nuclei in the 2-celled basidia, not simultaneous. 31. A mature 4-celled basidium with one newly formed epibasidium. 32. Epibasidium tipped by young basidiospore. 33, 34, 35. Basidiospores germinate by repetition. 36. Basidiospore germinating by producing secondary spores arranged in a fan-shape. 37, 40. Nuclei and cytoplasm in the cells moving into the epibasidia and basidiospores forming at the tips. 38. Each nucleus in the cell has divided and one daughter nucleus has migrated through a short sterigma into the basidiospore. 39. Basidiospore germinating by budding.

There is no evidence for the fusion of nucleoli during karyogamy as reported by Sappin-Trouffy (**17**) in *Auricularia auricula* and by Baker (**1**) in *Helicogloea,* nor for the degeneration of one of the nucleoli as reported by Olive (**13**) in *Coleosporium vernoniae.* Olive (**13**) and Sanwal (**16**) reported in *Coleosporium vernoniae* and *C. sidae,* respectively, that during prophase the nucleolus begins to become vacuolated, the vacuoles appearing first in its central portion. Then the outer region of the nucleolus collapses and becomes closely appressed to one side of the nucleus where it is seen as a flat, disc-shaped, darkly staining structure. In *Xenogloea,* the nucleoli are stained as deep purple dots by Hematoxylin. Frequently in the center of the nucleolus, there is a small spot which does not take the stain. It looks like the vacuole described by Olive and Sanwal. However, these vacuolated nucleoli are found in all stages of the development of the basidium and basidiospores (FIGS. 8, 22, 39). This seems to indicate that in *Xenogloea* the nucleolus is not solid; it is more probably a sphere with a hollow center. In *Xenogloea,* the nucleolus seems to have disappeared in the diplotene stage. However, no collapsed disc-shaped nucleolus such as Olive and Sanwal report has been seen. Besides, the nucleolus seems to reform or reappear in early telophase.

The presence of astral rays in *Coleosporium helianthi* and *C. vernoniae* as reported by Olive (**12, 13**), suggested that the astral rays probably assist in cross-wall formation. Sanwal supported Olive's view and stated that frequently at about the region of the intersection of the astral rays a large number of small vacuoles merge with one another and it is probable that the wall is the result of a coalescence. As a result of this, basidial cells may have their walls separated from one another by a space. Astral rays probably assist in accumulating some fluid cytoplasm around the vacuole, where it secretes a wall. In the present study, no astral rays were observed in *Xenogloea.* However, in a few cases, a vacuole-like space appears between two sets of chromosomes in the probasidium at telophase. In the mature basidium, the two cells separated from one another by a space have been frequently, but not always, found in the basidia on the leaf surface. This indicates that the formation of a space separating walls from one another is not connected with astral rays, as Olive and Sanwal suggest. The space might be due to plasmolysis of the cytoplasm during the fixation process.

The dark lines in certain figures of *Xenogloea* are similar to those in *Schizosaccharomyces;* and to the "rod-shaped spindle" in *Hyalopsora.* However, it is difficult to determine whether they are spindles or com-

posed of chromatin materials. It is possible that spindle fibers coalesce into one main strand. It also might be that they are chromatin materials since they are as darkly stained as the chromosomes.

The anaphase figure of *Xenogloea* is similar to that of *Auricularia mesenterica* (**9**), *Jola javensis* (**4**), *Helicogloea lagerheimi* (**1**) and many others. The telophase is similar to that of *Auricularia mesenterica* (**9**), *Schizosaccharomyces octosporus* (**5**), *Platygloea nigricans* (**11**), *Hyalopsora aspidiotus* (**14**), and *Helicogloea lagerheimi* (**1**).

The interpretation of the development of the basidia in *Xenogloea* is rather difficult. Although there is no evidence to prove that the hyphae growing out from the probasidium in the leaf will develop young basidia at their tips after reaching the stoma, or to show that the nucleus migrates through the hypha, yet the available information strongly suggests that the interpretation of the development of the basidium discussed previously is reasonable. However, it is doubtful whether it is possible for this diploid nucleus to pass through such a narrow hyphal part while undergoing meiosis without being disturbed. FIG. 16 shows a mass of chromatin material passing through the narrow hyphal part of the probasidium. This is evidence supporting the above interpretation.

In conclusion, *Xenogloea* is undoubtedly a specialized parasitic fungus in the Auriculariaceae. From the viewpoints of morphology and cytology, the structure and development of the probasidium and basidium in *Xenogloea* are different from those of other parasitic fungi in this family. It has its own special mode of development; thus it is a distinct genus in the Auriculariaceae.

SUMMARY

The application of Heidenhain's Hematoxylin and Trypan Blue staining techniques for the cytological study of *Xenogloea eriophori* suggests the following conclusions:

1. Clamp-connections and probasidia are found abundantly on the hyphae inside of the leaf of *Scirpus atrovirens* instead of being restricted to the pustules on the leaf surface.

2. Stages in meiosis in the probasidia of *Xenogloea* can be observed. The newly fused nucleus increases in size. Only one nucleolus is found in each nucleus after early prophase. There is evidence neither for the fusion of nucleoli during karyogamy, nor for the degeneration of one of the nucleoli. The nucleolus of *Xenogloea* is not solid; it is probably a sphere with a hollow center. Nucleoli seem to have disappeared in the diplotene stage and reappear in early telophase. The nuclear mem-

brane cannot be distinguished at diplotene stage. Chromosome number in metaphase I is eight. No astral rays are found.

3. The development of the basidia can be summarized as follows: Karyogamy occurs in the probasidium in the leaf and is soon followed by meiosis. Meiosis is completed in the probasidium in the leaf and a mature 4-celled basidium is formed there, or, for most of the probasidia, while the nucleus is in early prophase a hypha grows out at the basal or central portion of the probasidium, and grows toward the stoma. As soon as it reaches the stoma, a saccate structure is developed at its tip. The saccate structure increases in size; in the meantime, the nucleus in the probasidium in the leaf migrates through the hypha into the saccate structure in which meiosis is completed. A 4-celled basidium is thus formed in the pustules on the lower surface of the leaf. The basidiospores germinate by repetition, by budding or by producing one to four secondary spores, arranged, if more than one, in a fan-shaped pattern.

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NOTES ON BOLETES. IX

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Report is here made of certain matters of some importance and of others of minor significance.

NEW SPECIES

In 1949, Mrs. Phyllis Gardner McMillan, then Herbarium Botanist at the University of California, sent some specimens from frequent collections by Mrs. Morton R. Gibbons at Huntington Lake, Fresno County, California, with notes on fresh and dried material. Mrs. Gibbons herself sent abundant material later.

The carpophores are noteworthy for their squat appearance, their short stipes, and especially the coarse hymenophore with very large "tubes" and very thick, irregular veins or lamellae. The hymenophore and stipe are glandular-dotted, the stipe thickly so to the base. A veil covers the hymenophore when young and breaks to form a thin but ample annulus which later may entirely disappear.

Suillus megaporinus sp. nov.

Carpophoro corporis brevis atque lati, brevi-stipitato, late turbinato. Pileo e subdepresso plano, plus minusve viscido vel subviscido, glabro, fortasse appresso-tomentoso, plus minusve fibrilloso vel fibrilloso-squamuloso, e pallide flavido avellaneo, fibrillis subferrugineis, 2–7 cm lato. Carne pallide ferrugineo-avellanea, immutabili. Hymenophoro profunde decurrenti, boletinoideo cum lamellis crassis atque "tubulis" amplis glandulosisque. Stipite brevissimo, e pubescenti tomentoso, fortasse scabroso, dense glanduloso-punctato, annulato, concolori, 8–20 × 5–7 mm. Sporis in cumulis subolivaceis, sub lente e hyalinis pallidissime flavido-viridibus, ellipticis, (5–)7–10 × 3.3–3.8 μ.

Carpophore squat, short-stipitate, broadly turbinate. *Pileus* plane to somewhat depressed, occasionally subumbonate, 2–7 cm broad. *Surface* apparently subviscid, if not viscid, glabrous or perhaps in places appressed-tomentose, with or without scattered fibrillose or fibrillose-squamulose ornamentations; yellowish to pale yellowish-tan or avellaneous, the fibrils or scales reddish-brownish, when dry between Cream Buff and Chamois, darker and dingy reddish-brownish when old. *Flesh* pale reddish-tan, unchanging. *Hymenophore* deeply decurrent, boletinoid to coarsely lamellate-tubulose with thick lamellae or veins and large compound "tubes" up to 5 mm long, with the walls and edges

glandular-dotted, and the openings or "pores" up to 7 or rarely 10 mm radially and 1 mm or more broad; bright yellow becoming rusty with age. *Stipe* central to more or less eccentric, very short, tapering downward; tomentose to pubescent, perhaps minutely hispid, perhaps more or less scabrous, thickly glandular-dotted to base, concolorous, the tomentum at base buff to rusty-orange; 8–20 × 5–7 mm below and larger upward perhaps to 1 cm. *Veil* covering the tubes when young, contracting where the tubes terminate on the stipe to a flaring, membranous annulus, which may disappear entirely or may leave remains variously membranous or fibrous on the stipe or on the lower tubes. *Spores* perhaps somewhat olivaceous in deposit, hyaline to very faintly yellowish-greenish, elliptical, (5–)7–10 × 3.3–3.8 μ, mostly 8–8.5 × 3.5 μ. *Cystidia* of two kinds, in the hymenium hyaline, clavate to lanceolate, 25–50 × 4–5 μ, and in the glandular dots, yellowish to brownish, irregularly clavate to irregularly wrinkled or undulating, 50–105 × 5 μ.

In grass or moss at the edge of mountain meadows near *Pinus contorta* and *Abies concolor* at 7000 feet, at Huntington Lake, Fresno County, California. Late August and early September.

The TYPE collection is No. *2111* in WHS Bolete Herbarium, and there are specimens in the Herbarium of the Department of Botany of the University of California.

The chemical reactions of dried material are as follows: NH_3—pileus more or less violet to violet-gray or brownish, flesh faintly rosy to dark lilac, tubes definitely red, stipe more or less so; acids—pileus and tubes orange and flesh orange to reddish; KOH—flesh deep lilac and tubes red; $FeSO_4$—pileus somewhat bluish-gray and flesh possibly somewhat so.

This species apparently belongs in Singer's Subsection Hirtellini of the Section Granulati (**1**, pp. 259 and 273).

In Singer's Subsection Angustiporini of the Section Granulati of the genus *Suillus* (**1**, pp. 259–260), there have been known four species with a veil that forms a distinct annulus. Two of them, *S. acidus* (Pk.) Sing. and *S. subluteus* (Pk.) Snell, occur in association with *Pinus Strobus*. *S. luteus* (L. ex Fr.) S. F. Gray has long been known as associated with the Series Lariciones of the subgenus Diploxylon of the genus *Pinus*. Then recently Singer named *S. cothurnatus* as associated with the southern pines of the Series Australes.

There has for some time been evidence of the existence of a *luteus* complex. It has at times appeared that there might be subspecies or forms associated with *Pinus sylvestris* and *P. resinosa*, and even segre-

gates of different appearance under *P. resinosa* alone. Further, one might mention the disturbing situation presented by collections which look like *S. luteus* stated to have been collected under or near *Picea* and *Abies*.

Whatever may be the situation with regard to the foregoing, the present writers last fall found a reasonable abundance of specimens in a mixed stand of *Pinus Strobus, P. rigida* and *Quercus* spp. but under conditions which plainly pointed to *P. rigida* as the tree-associate in every case. These carpophores had the general appearance of *S. luteus* but with the following pronounced differences: the carcophore on the whole not so stocky, the pileus not so thick or so convex as in *S. luteus*, and the stipe thinner; the olive colors of the pileus; the entire carpophore drying blackish; the orange colors of the context, hymenophore and stipe (especially the base); glandular dots not present on the tube walls but present on the pores and also on the ring of young tissue beyond the outer limit of the tubes, and on the edges of the thin, marginal roll.

Suillus pinorigidus sp. nov.

Carpophoro nigrescenti. Pileo plano vel plano-convexo, viscido, variatim flavido, Cinnamon Buff, Clay Color, Cinnamon, Tawny, Dark Olive Buff, Buffy Olive, Deep Olive, Deep Grayish Olive, vetusto olivascenti, sicco nigridio, 3–8 cm lato. Carne Pale Orange Yellow, Light Orange Yellow vel Orange Buff. Hymenophoro subdepresso, Capucine Yellow, Orange Buff, Mars Yellow vel fortasse Raw Sienna, postremo Mummy Brown; poris concoloribus, parvis. Stipite subaequali, recto vel curvato, basi acute curvato, dense glandulis adornato, annulato, plerumque Capucine Buff, supra annulo griseolo, basi Salmon Orange; intra sursum concolori, deorsum e Orange Xanthine Orange; 3–7 × 8–13 mm. Sporis in cumulis novis Honey Yellow vel Clay Color, aliquanto post Honey Yellow, Isabella Color vel Buckthorn Brown, ellipsoideis, sub lente pallidis, 7.5–10 × 2.5–3.5 μ.

Carpophore in its entirety tending to dry blackish instead of being destroyed by moulds, eelworms, etc. *Pileus* plane to plano-convex, the margin often in a thin roll which is glandular-dotted, up to 8 cm broad. *Surface* viscid, variously yellowish to Cinnamon Buff, Clay Color, Cinnamon or Tawny to between Dark Olive Buff and Buffy Brown, to almost Buffy Olive, Deep Olive, or Deep Grayish Olive with darker (almost black) streaks or fibrils, tending to have the olive tones when older, and drying blackish. *Context* Pale Orange Yellow, Light Orange Yellow, or Orange Buff, mostly the last; odor pleasantly fungoid, taste pleasant, perhaps very slightly farinaceous. *Hymenophore* slightly depressed around the stipe, dull Capucine Yellow to Orange Buff and later more Mars Yellow or perhaps Raw Sienna, finally Mummy Brown; tubes 5–8 cm long, pores concolorous, glandular-dotted, less than 1 mm

broad. *Stipe* subequal, straight or curved, almost always bent at a sharp angle at the very base, thickly glandular-dotted almost to base, annulate (sometimes as low as half-way down), mostly Capucine Buff becoming grayish above the annulus, the basal portion almost Salmon Orange becoming a dull Salmon Orange, the glandular dots at first Ferruginous becoming almost black on the upper portion of the stipe and brownish below; within like the context in the upper portion but the lower half between Xanthine Orange and Orange, with these colors continuing up the rind; 3–7 cm long × 8–13 mm thick. *Veil* covering the hymenophore, membranous and fairly tough, whitish, brown to blackish at stipe, rupturing to form an annulus up to 4 mm wide when fresh, whitish with a dark band in the middle, later collapsing to a gray band dark brown inside, finally entirely black. *Spores* in deposit between Honey Yellow and Clay Color when fresh, later between Honey Yellow and Isabella Color, or Buckthorn Brown, elliptic-subfusiform to cylindrical, hyaline to very pale greenish, 7.5–10 × 2.5–3.5 μ. *Cystidia* mostly clustered, obtuse, subcylindric, more or less undulating, hyaline, more rarely clavate or cylindric-clavate or fusoid, 21–35 × 3–9 μ; dermatocystidia of glandular dots, undulating-cylindrical to clavate, brown, more or less encrusted, 35–65 × 5–9 μ.

Under *Pinus rigida*, Scituate, R. I.
TYPE collection WHS Bolete Herbarium No. *2112*.

We reported previously (**6**, pp. 24–25) that we were certain that we had a collection of *Boletus variegatus* Swartz ex Fr. from Middleboro, Massachusetts, inasmuch as the only character different from the European descriptions was the large pores. We were wrong, for there is another difference—larger spores. Therefore, it now appears that this collection is not the European *variegatus*, but should be described as new.

Since *B. variegatus* is now considered a *Suillus* [*S. variegatus* (Sw. ex Fr.) Kuntze] and since our specimens so much resemble this species, we are considering them to be *Suillus* in spite of the lack of a pronounced viscidity of the surface (as far as we now know). In Singer's arrangement (**1**, pp. 259 and 278), *S. variegatus* is placed in the Section Bovini, Subsection Stenoporini, characterized by narrow pores (1 mm or less broad), squamulose pileus and indistinct reaction of the flesh with NH_3, whereas the Subsection Euryporini is characterized by large pores, non-squamulose pileus and rosy or red reaction of the flesh with NH_3. Our species accordingly will not fit properly in either Subsection because, outside of lack of information on the reaction with NH_3, the pileus is squamulose but the pores are very broad.

Suillus subvariegatus sp. nov.

Pileo plano-convexo, e sicco humido, tempestate pluviosa subviscoso, e fasciculato-piloso fibrilloso-squamuloso, subflavo vel subaurantiaco, squamulis fuscis, 5–12 cm lato. Carne e pallide flavescenti pallide aurantiaca, subimmutabili vel rarius coerulescenti. Hymenophoro primo pallide flavo, deinde clariori et subaurantiaco, mox plus minusve olivaceo et postremo flavobrunneo, fortasse viridi-cyanescenti, poris latissimis. Stipite subaequali, e furfuraceo fibrilloso, flavo, saepe plus minusve subrubro praecipue basi. Sporis in cumulis e olivaceis subbrunneis, subelliptaceis, 9–14 × 3.5–5 μ.

Pileus plano-convex to plane, 5–12 cm broad. *Surface* dry to moist or subviscid, somewhat slimy in wet weather, covered with dark, superficial, fasciculate, hairy squamules that may entirely disappear in the rain or in age; at first yellowish-gray or pale orange, gradually becoming yellowish-brown to orange-yellow, occasionally tinted with olive or reddish; scales darker than the ground color, yellowish, reddish-brownish or even blackish. *Flesh* yellowish to pale orange, lemon-yellow above the tubes, becoming darker, changing to bluish-green or not at all changing; odor somewhat unpleasant, often disagreeable; taste not noteworthy. *Hymenophore* adnate to subdecurrent, at first pale yellow, later brighter and more orange, soon more or less olivaceous and finally rich yellow-brown, perhaps becoming bluish-green when wounded; pores concolorous, angular, large, 1.5–4 mm broad. *Stipe* subequal, furfuraceous at the base, for the most part fibrillose to almost fibrillose-squamulose and often more or less pseudoreticulate from the fibrils; pallid to pale yellow or more or less bright yellow, often more or less reddish, especially at the base; within yellowish above, brighter under the cortex, orange-yellow to brownish or even reddish toward the base; 4–7 cm long, 10–20 mm thick. *Spores* olivaceous in deposit when fresh, brownish without olive tone when dry, subelliptical to elliptical-subfusiform, almost hyaline to very pale greenish, 9–14 × 3.5–5 μ, mostly 11–12 × 4–4.5 μ. *Cystidia* few, clavate, hyaline, 30–55 × 8–10 μ. Hyphal structures in mounts of hymenium hyaline, with subclavate or variously pointed tips, 80–265 × 7–11 μ.

Under white pine with admixture of oaks, Middleboro, Massachusetts.

TYPE collection WHS Bolete Herbarium No. *232*.

CERTAIN OF THE LURIDI

With or without Singer's complete and excellent treatment of the Luridi (**2**, pp. 56–86), collectors continue to have difficulties with and misconceptions concerning what may be called the *luridus-erythropus-subvelutipes* complex. Many persist in calling everything in this group

B. luridus Schaeff. ex Fr. whether specimens have the reticulate or the furfuraceous stipe. Many also fail to distinguish the rather common, eastern *B. subvelutipes* Pk. from *B. erythropus* (Fr. ex Fr.) Pers., the occurrence of which in this country is still uncertain (see **5**, pp. 379–380). It is only fair on the other hand to say that in any event there are difficulties in this group and some situations puzzling even to close students.

In the common understanding and in most of the treatises, any specimen in this complex with a reticulate stipe must be *B. luridus;* a specimen with the stipe furfuraceous or flocculose-specked is one of the other two species. Singer (**2**) states on page 59 that *B. luridus* is a "European species, probably not occurring in America," and on page 81 that *B. luridus* of Coker and his school is *B. miniato-olivaceus* Frost var. *subluridus* (Murr.) Sing., with a further question on page 68 of the relation to *B. hypocarycinus* Sing. We have in general been inclined to agree with Singer that the common European *B. luridus* does not occur on this continent, except for one item which is to follow, even though we have occasionally collected specimens that looked like *B. subvelutipes* but which had stipes more or less reticulate. We think we have an explanation of this latter situation.

A few years ago, in a spot in Providence, R. I., where we have collected *B. subvelutipes* in some abundance in any favorable season, we found several specimens which were obviously this species. Four of these were in an area of about two square feet. Two of them were single and the other two had the bases of the stipes in contact although not fused, but with the pilei completely fused for almost the entire width. The stipes of one of the single specimens and of one of the pair with fused pilei were partially reticulate, finely but nevertheless plainly so. It would not be safe to say that all four of these specimens were produced from the same mycelium but it would be reasonable to raise a question as to the importance of this matter, because of the small area in which the specimens grew and the repeated occurrence of *B. subvelutipes* in this spot. Further, it would seem that there is no need to raise the question of the compatibility of the pileic substance in connection with the fusion of the pilei. It appears to be reasonable then to conclude that the two specimens with the fused pilei grew from the same mycelium, and that these carpophores were both *B. subvelutipes.* Furthermore, the reticulation of the stipes of the two carpophores was not sufficiently extensive nor coarse enough to suggest an identification as *B. luridus.*

So much for the specimens which look like *B. subvelutipes* and have

stipes reticulate with fine lines. Now for another situation, referred to above. Occasionally in the northeast we have seen specimens of Luridi obviously in this complex, which do not look like *B. subvelutipes* and have on the stipe a coarse reticulation which is rather strongly elevated and more or less red. *B. luridus* is immediately suggested. The carpophores resemble European specimens which we have in our collections and the characters appear to fit the European descriptions. One is always hesitant about making a definite identification as *B. luridus* but at least such material demonstrates that we have in this country either *B. luridus* or a species very close to it.

BOLETUS IMPOLITUS

Our good friend Rolf Singer (**2**, p. 79) feels that *Boletus impolitus* Fr. has "not yet (been) observed with sufficient certainty in North America," in spite of the announcement of the finding of two carpophores at Riverside, N. Y., in 1934 (**3**, pp. 14–15).

In 1935, in the same spot and also 50 yards away, by the roadside, the senior author found seven fully-developed specimens and also other specimens near Ausable Chasm, N. Y. (**4**, p. 463). In the same year three specimens were obtained at Deerhead, N. Y. Two collections were made again at Riverside in 1937, of which only one collection of three specimens was retained, and another specimen was found in the same spot in 1946. It would seem that this species fruits quite frequently, although never in abundance, in this spot. We have no uncertainty about the identification.

We have records of a collection by Thaxter at Kittery Point, Maine, now in the Farlow Herbarium, and also of a collection in the Herbarium of the University of Connecticut from Mansfield. We have not seen these specimens.

LECCINUM AURANTIACUM

Of *Leccinum aurantiacum* (Bull. ex) S. F. Gray, Singer says (**2**, p. 123): "This somewhat variable species is most frequent in Europe and America under or near *Populus tremula* and *P. tremuloides*. It is more rarely found under other trees," and also (**2**, p. 119): "mycelium associated with *Populus, Carpinus, Fagus* and *Quercus*."

We find this under *P. tremuloides* and possibly other species of *Populus* in the northeastern states and eastern Canada. We have also found it in the following situations and locations: one collection under *Pseudotsuga taxifolia* (*Menziesii*) at Potowomut, R. I.; under *Pinus*

rigida and *Arctostaphylos Uva-ursi* in Rhode Island; in pure stands of *Pinus rigida* with no *Arctostaphylos* nearby, at Centerville, Massachusetts (see **5**, p. 375); regularly on the bank of a road-cut near Sandwich, Massachusetts, in a dense growth of *Arctostaphylos Uva-ursi* with no pitch pine near and only *Prunus* sp. at the top of the bank; one collection in Providence, with *Betula lenta* and *Quercus* spp. nearby.

One of the strangest occurrences of what apparently obviously was this species of bolete was some specimens from an abundant collection made by Dr. J. B. Carpenter of the USDA Cooperative Rubber Plant Field Station, Turrialba, Costa Rica, on Volcan Irazu, at an altitude of 3432 meters. Unfortunately, Dr. Carpenter has as yet been unable to furnish the identity of the trees and shrubs near which the fungus grew.

LECCINUM CHROMAPES

Singer says (**2**, p. 124) that *Leccinum chromapes* (Frost) Sing. occurs under *Betula, Populus* and *Salix*.

Our most recent specimens were collected in 1954 near the Seekonk River in Providence under *Betula lenta* and *Quercus* spp. We have found it often near Warrensburg, N. Y., in a stand of *Quercus* spp. without any *Betula*, frequently at Riverside, N. Y., in a stand of *Tsuga canadensis* with no *Betula* within a reasonable distance (only one *Betula populifolia* in the stand), and in many other localities in which hemlock predominated and birch was scattered, scarce, or entirely lacking. We have also found this species at Friendship, Maine, in a stand of *Picea glauca, P. rubens* and *Abies balsamea* at considerable distance from the two or three birches and poplars in the stand.

BOLETUS REGIUS

S. Billeci sent us some fruit bodies collected on the day after Christmas in 1954 "under tan oak, live oak, red oak and madroña" in a canyon 10 miles north of Santa Cruz, California, elevation 200–300 feet. It is one thing to name as a new species California material which is conspicuously and completely different from anything described, but common prudence dictates great caution in determining with certainty west coast specimens to be the same as a well-known European species. Billeci's specimens do, however, fit in every particular the descriptions and illustrations of *Boletus regius* Krombh. of Europe.

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DACRYMYCES PALMATUS

LORENE L. KENNEDY [1]

(WITH 3 FIGURES)

In October 1954 a collection labelled *Dacrymyces palmatus* (Schw.) Bres. was received from Dr. B. Lowy which exhibited two interesting features not usually associated with this species. First, the fungus was found growing on angiosperm wood and descriptions state that this species grows on gymnosperm wood. Second, the stalk, where present, and the radicating base of the fused basidiocarps showed a very striking development of cortical (surface) hairs. Subsequent collections made in the same area, Baton Rouge, Louisiana, were similar. In all other respects these collections agreed with published descriptions of this species. These specimens raise the question whether they represent a variety of *Dacrymyces palmatus* growing on angiosperm wood and modified because of the substratum.

The species was originally described by Schweinitz in 1832 as *Tremella palmata*. In 1904 Bresadola transferred it to *Dacrymyces*. It should be noted here that according to Lloyd (Myc. Writ. **6**: 989. 1921) *Tremella aurantia*, described by Schweinitz in 1822, and deposited in his herbarium, is not a *Dacrymyces* but a *Tremella*. Hence *Tremella aurantia* should not be included in the synonymy of *Dacrymyces palmatus* and the name *Dacrymyces aurantius* (Schw.) Farl. is invalid. Lloyd, in 1921, placed a form of *Dacrymyces palmatus* in the genus *Dacryopsis* because of its distinct head and stalk, and called it *Dacryopsis palmata*. This name was based on Ellis & Everhart N.A.F. *1697*, a collection distributed as *Tremella aurantia* Schw. It appears that different material was distributed under this number, for, while Lloyd's collection seems to be *Dacrymyces palmatus*, judging by the figure and description, the material in the S.U.I. herbarium is *Tremella lutescens* Fries. This situation has added further to the confusion concerning the species referred to as *Tremella aurantia* Schw. In 1935 Wehmeyer described a form of *Dacrymyces palmatus* under the name *Dacryomitra ramosa* and it is evident that his collections, like those of Lloyd, were markedly stipitate. Kobayasi (Sci. Rep. Tokyo Bunr. Daig. B. **4**: 119. 1939) uses the name *Dacrymyces aurantius* (Schw.) Farl. for a species

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segregated from *Dacrymyces palmatus* by its "pulvinate form, cartilaginous consistency and thick-walled peridial hairs." These collections appear, from the description, to be the sessile form of *Dacrymyces palmatus* with well-developed cortical hairs.

Although *Dacrymyces palmatus* may be considered a well-known species, the various descriptions differ considerably as to details of its structure. It is apparent from the literature that the species must show a considerable range of variation in many of its features, especially those concerned with external form. This would be responsible for the differing descriptions and for the extensive synonymy. Taken all together, the various descriptions incorporate practically all the detail required for a complete description: however, no single account gives an adequate picture of the range of features found in the species.

Study of herbarium material revealed a notable variation in many of the characters and these will be described and discussed from the standpoint of their value in identification.

1. *Substratum.* The great majority of the collections were on gymnosperm wood; however, five collections were on angiosperm wood. It may be concluded that although corticate or decorticate gymnosperm wood is the common substratum, this fungus will grow on angiosperm wood. Since this is the case, not only with *Dacrymyces palmatus,* but with other species of this genus, it is inadvisable to use the type of substratum, without qualification, as a character in keys to the species *Dacrymyces.*

2. *Macroscopic Features.* The color is a striking bright orange when fresh or soaked, and orange-red when the basidiocarps are dry. Color is often thought to be an unreliable taxonomic feature, but the color of this species is one of its outstanding external features in both fresh and dry conditions. It is lost only upon repeated soaking of the material.

Dacrymyces palmatus shows an extensive range in form which is chiefly responsible for the number of synonyms (FIGS. 1, 2). It may occur as a sessile, pulvinate or flabellate basidiocarp, as stalked and spathulate or as stalked with a discoid, convoluted or cupulate head. More often, a number of basidiocarps have coalesced to form a cerebriform mass, an erect, petaloid mass, or a structure composed of a compound fused stalk and fused pilei. Whether there be one basidiocarp or a cluster, there is always a distinct, white, radicating base penetrating the wood. Since the sporocarps may be simple or compound, measurements mean little unless the kind of structure measured is stated. In the material studied single basidiocarps measured up to 7 mm wide by 5 mm

high, while compound basidiocarps measured 3 to 6 cm in longest dimension. As this species ranges from completely sessile through to apparently stipitate and pileate, it appears that the character of sessile, stipitate or substipitate is not a sufficient basis for distinguishing the genera *Dacrymyces, Dacryomitra* and *Ditiola*. Furthermore, all these genera contain species with distinct rooting bases penetrating the wood. There-

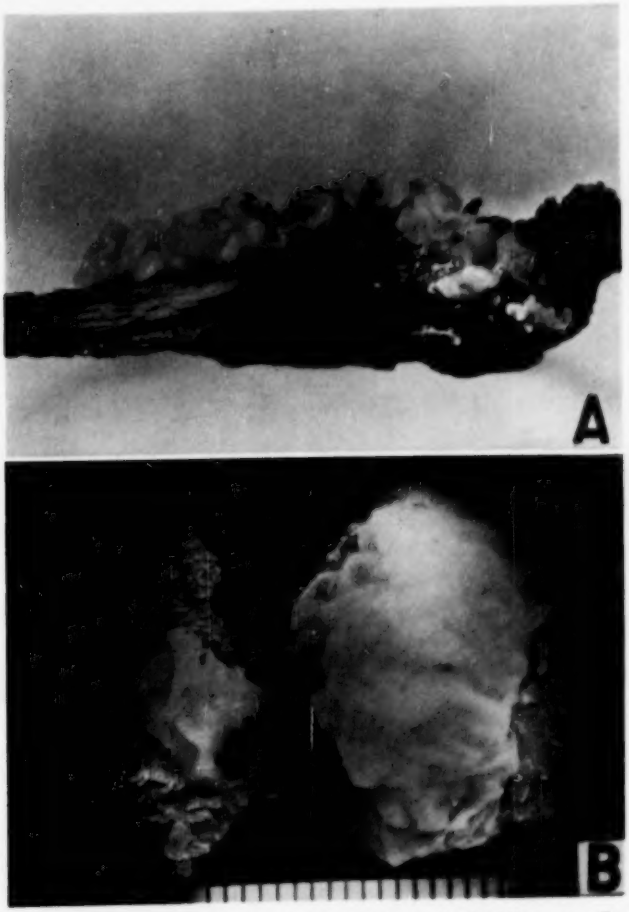

FIG. 1. A. Cerebriform mass of coalesced, sessile basidiocarps. B. Single cupulate basidiocarp and a compound structure of fused, cupulate basidiocarps, growing side by side. All approximately × 2½.

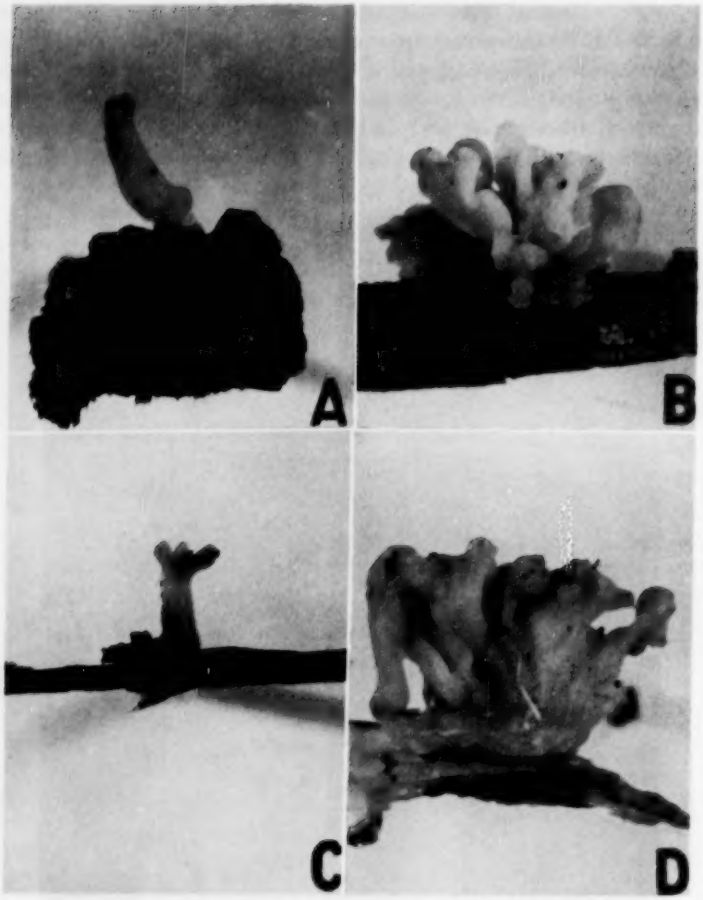

FIG. 2. A. Single, stalked, spathulate basidiocarp. B. Petaloid cluster of spathulate basidiocarps. C. Single, stalked and pileate basidiocarp. D. Compound structure of fused, pileate and stalked basidiocarps. All approximately × 2½.

fore, it is suggested that a true stipe be defined as a stalk which projects beyond the bark and which is composed of thick-walled hyphae as contrasted with the thin-walled hyphae of the pileus. In other words the texture is heterogeneous. This is the condition found in *Ditiola radicata* Fries and in *Dacryomitra nuda* (Berk. & Br.) Pat. In the substipitate form of *Dacrymyces palmatus* the stalk usually does not project far beyond the bark; where it does, or where the bark is absent, the stalk

is composed of the same thin-walled hyphae as the pileus. That is, the texture is homogeneous.

As Nannfeldt (3) has pointed out, the internal structure of the basidiocarps of the species in the Dacrymycetaceae has been neglected

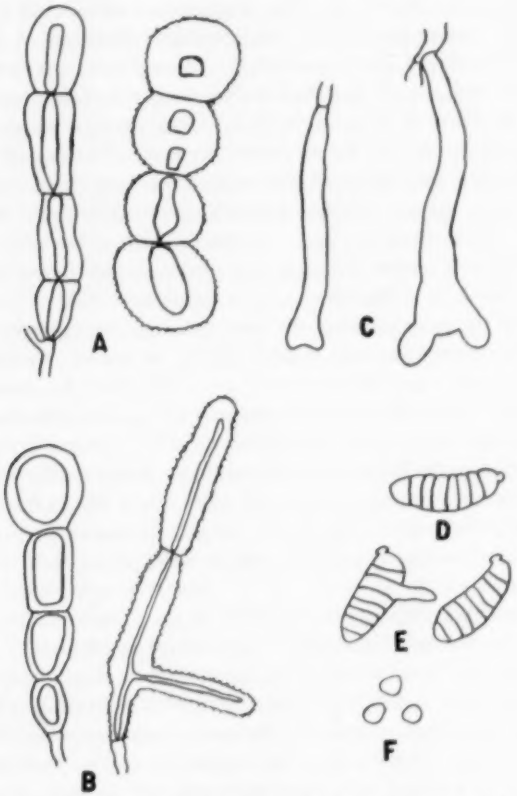

FIG. 3. A. Cortical hairs from collections on angiosperm wood. B. Cortical hairs from collections on gymnosperm wood. C. Stages in basidial development at time measurement should be made. D. Spore from collection on gymnosperm wood. E. Spores from collections on angiosperm wood, one with germ tube. F. Conidia. All approximately × 1000.

and too much reliance has been placed on external shape, which is very variable. Further study along these lines is desirable and might lead to a more natural grouping of the species than now exists. For example, *Dacryopinax spathularia* (Schw.) Martin is composed entirely of

thin-walled hyphae while *Dacryopinax elegans* (Berk. & Curt.) Martin has thick-walled hyphae in both pileus and stalk; *Dacryomitra nuda* (Berk. & Br.) Pat. is heterogeneous, with thick-walled hyphae in the stalk and thin-walled hyphae in the pileus, while *Dacryomitra stipitata* (Peck) Burt is homogeneous, with thin-walled hyphae throughout.

3. Microscopic Features. The development of cortical hairs on the substipe and rooting base of the Baton Rouge collections of *Dacrymyces palmatus* has already been mentioned. These hairs are long, septate, cylindrical or clavate, thick-walled with a narrow lumen, often appearing somewhat moniliform (FIG. 3, A, B). They may be rough or smooth and break off easily from the parent hyphae. Studies of herbarium material showed that most of the collections had these cortical hairs developed to a varying degree, regardless of substratum, while a few specimens lacked them entirely. Certainly, the collections on angiosperm wood show a more constant and extensive development of cortical hairs than do most collections on gymnosperm wood. However, it is believed that the description of the species should be extended to include this feature rather than that a new species or variety should be based upon it. In this connection it may be noted that the character of a differentiated cortex is used to separate the genera *Guepiniopsis* and *Femsjonia* from the genus *Dacrymyces*. This cortex is composed of thick-walled, septate hyphae which are very similar to the surface hairs of *Dacrymyces palmatus*, *Femsjonia luteoalba* Fries, especially, could be confused with some forms of *Dacrymyces palmatus*, particularly with those on angiosperm wood. In such a case there is a similar substratum, similar cortical hairs, spores about the same size, shape and septation, and a basidiocarp of similar form. Since cortical hairs are found in species of *Dacrymyces*, *Ditiola*, *Guepiniopsis*, *Femsjonia*, *Dacryopinax* and *Dacryomitra*, it appears that this feature by itself should not be used as a leading character in the separation of the genera.

In the collections examined, clamp-connections were rarely seen, although bulbous septa and what appeared to be collapsed clamp-connections were found. In most specimens the hyphae were definitely septate. It is thought that clamp-connections may be present at an early stage, since bulbous septa and collapsed clamp-connections would suggest this condition. The internal hyphae of the basidiocarp may have either a rough or smooth surface; sometimes both kinds occur in the same basidiocarp. Hence this character is not constant within the species.

In some of the descriptions of the species belonging to the various genera of the Dacrymycetaceae basidial measurements are given, but often it is not clear what is meant by the term basidium. The authors

do not state at what stage in the development of the basidium measurements were taken. It is known that the length of the mature basidia will vary according to the thickness of the basidiocarp, since one of their functions is to elevate the nuclear material to the surface of the basidiocarp where the spores will be formed. In a relatively dry season the basidia will be shorter than when moisture is abundant and a thicker basidiocarp is formed. The basidium is at first cylindrical, then clavate and finally produces two extensions or epibasidia. The epibasidia elongate until the surface of the basidiocarp is reached and then form short sterigmata upon which the spores are borne (**1**). Since the length of the epibasidium varies with the thickness of the basidiocarp, which is, in turn, dependent on external conditions, measurements of, or including, the epibasidia are of little taxonomic value. However, the length of the probasidium is not greatly modified by the thickness of the basidiocarp and it is suggested that this may be a character of taxonomic significance. The probasidium is considered to be the basidium as epibasidia are beginning to form and its length the distance between the basal septum and the tip of the central axis (FIG. 3C). Measurements were made of the probasidia of *Dacrymyces palmatus* collections and the majority measured 50–70 μ although the range extended from 45 to 80 μ. Kobayasi (Nagaoa **4**: 36–47. 1954) may have had something of this sort in mind when, in the description of his new species *Dacrymyces albidus*, he gives hypobasidial and epibasidial measurements. Although he does not state at what stage of maturity these measurements were taken, it is likely that the hypobasidial length would be similar to the probasidial length as described above.

The spores are subject to less variation than any other feature (FIG. 3D, E). They are orange in mass, curved-cylindrical and distinctly apiculate. Although at first continuous, they early become 7-septate. The number of septa is constant and is one feature which serves to distinguish this species from *Femsjonia luteoalba*, which has a large, but varying number of septa, often more than seven. It was found that spores varied little among the different collections, those of nearly all collections measuring 17–21(–25) × (4–)6–7(–8)μ. They germinate by oval conidia or by germ tubes; sometimes the two modes of germination are found in the same preparation.

The study of this one species bears out Neuhoff's (**4**) contention that the genera of the Dacrymycetaceae are connected with one another through transitional forms. It appears true also that no single feature can be used to separate either the genera in this family or the species within the genus *Dacrymyces*.

The following description will allow for the range of variation found in this species.

DACRYMYCES PALMATUS (Schw.) Bres. in Hoehn. Oesterr. Bot. Zeit. **54**: 425. 1904.

?*Naematelia rubiformis* Fries, Syst. Myc. **2**: 228. 1823.
Tremella palmata Schw. Trans. Am. Phil. Soc. II. **4**: 186. 1832.
Dacryomyces chrysospermus Berk. & Curt. Grevillea **2**: 20. 1873 (as *chrysosperma*).
Dacryomyces aurantius Farl. Appalachia **3**: 248. 1883 (as *Dacryomyces aurantius* (Schw.) Farl.
Dacryomyces multiseptatus Beck, Fl. Hernstein 126. 1886.
Dacryomyces flabellus Ell. & Ev. Proc. Acad. Nat. Sci. Phila. **46**: 324. 1894 (*as flabella*).
?*Dacryomitra depallens* Lloyd, Myc. Writ. **5**: 803. 1918.
Dacryopsis palmata (Schw.) Lloyd, Myc. Writ. **6**: 989. 1921.
Dacryomyces palmatus (Schw.) Burt, Ann. Missouri Bot. Gard. **8**: 361. 1921.
Dacryomitra ramosa Wehmeyer, Papers Mich. Acad. Sci. **20**: 249. 1935.
?*Dacrymyces rubiformis* (Fries) Neuh. Arkiv. för. Bot. **28A**(1): 51. 1936.

Bright orange, drying orange-red; tough-gelatinous, becoming soft and collapsing when old; conspicuous when dry; basidiocarp sessile, pulvinate or flabellate, or stipitate, with a spathulate or convoluted, patellate or cupulate head, up to 7 mm wide, 5 mm high; attached by a white, radicating base; commonly erumpent in clusters which coalesce to form an erect, petaloid, cerebriform or stalked and pileate compound sporocarp from 3 to 6 cm in longest dimension; substipe and rooting base with a white cortex composed of cylindrical or clavate, septate hyphae with thick, gelatinized walls, often rough on the surface, up to 80 μ in length and 7 μ wide (these apparently lacking in a few collections); internal hyphae thin-walled, smooth or rough, loosely interwoven, septate, occasionally with bulbous septa or clamp connections; probasidia (45–)50–70(–80) × 3–6 μ with basal septa; basidiospores orange in mass, curved-cylindrical, apiculate, distinctly 7-septate, 17–21(–25) × 6–7(–8) μ, germinating by oval conidia or by germ tubes.

TYPE LOCALITY: Bethlehem, Pennsylvania.

HABITAT: Corticate and decorticate gymnosperm and angiosperm wood.

DISTRIBUTION: Temperate North America; eastern Asia; Europe.

ILLUSTRATIONS: Jour. Mitchell Soc. **35**, pl. 23, f. 10; pl. 63, f. 6, 7; Farlow, Icon. Farl. pl. 100; Bres. Icon. Myc. **23**, pl. 1126, f. 2; Papers Mich. Acad. Sci. **20**: 249, f. 3; Am. Midl. Nat. **20**: 231, f. 42–45; Sci. Rep. Tokyo Bunr. Daig. B. **4**, pl. 11, f. B.

Single, cupulate or discoid, substipitate basidiocarps of this species growing on angiosperm wood bear a close resemblance to *Femsjonia luteoalba*. However, in *Femsjonia luteoalba* the spores are tardily 3–12-septate; the hyphae and probasidia bear conspicuous clamp-connections and, in my experience, clusters of basidiocarps are not found, although two or three may fuse partially. Furthermore, the cortical hairs have clamp-connections and generally appear to be thick-walled hyphae rather than distinct, cylindrical or clavate structures. The large 7-septate spores serve to separate some forms of *Dacrymyces palmatus* from species of *Dacryomitra* and *Dacryopinax;* the spores and homogeneous texture from species of *Ditiola*.

SUMMARY

1. The variations in form and microscopic features of *Dacrymyces palmatus* are described.

2. Using these features as examples, the inconstancy of many characters found in keys to the species of *Dacrymyces* and to the genera of the Dacrymycetaceae is discussed.

3. A description is presented which, it is believed, will allow for the range in external form and microscopic features exhibited by this variable species.

This study was carried out under the direction of Professor G. W. Martin.

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OCCURRENCE OF TRICELLULA INAEQUALIS IN THE UNITED STATES

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(WITH 6 FIGURES)

In the course of a survey of airborne molds in the vicinity of Atlanta, Ga. (1), two isolates of an apparently undescribed fungus were obtained on exposed "gravity" plates of Czapek-Dox agar. The two were taken at different times and at different sites, the first on October 28, 1953 in the downtown area and the second on February 17, 1954 in the suburb of Chamblee. Meteorological conditions in the two instances were similar, as is indicated in TABLE I.

As observed on the original plates, the colonies were pale orange, moist, and glabrous. The septate mycelium bore numerous tricornate,

TABLE I

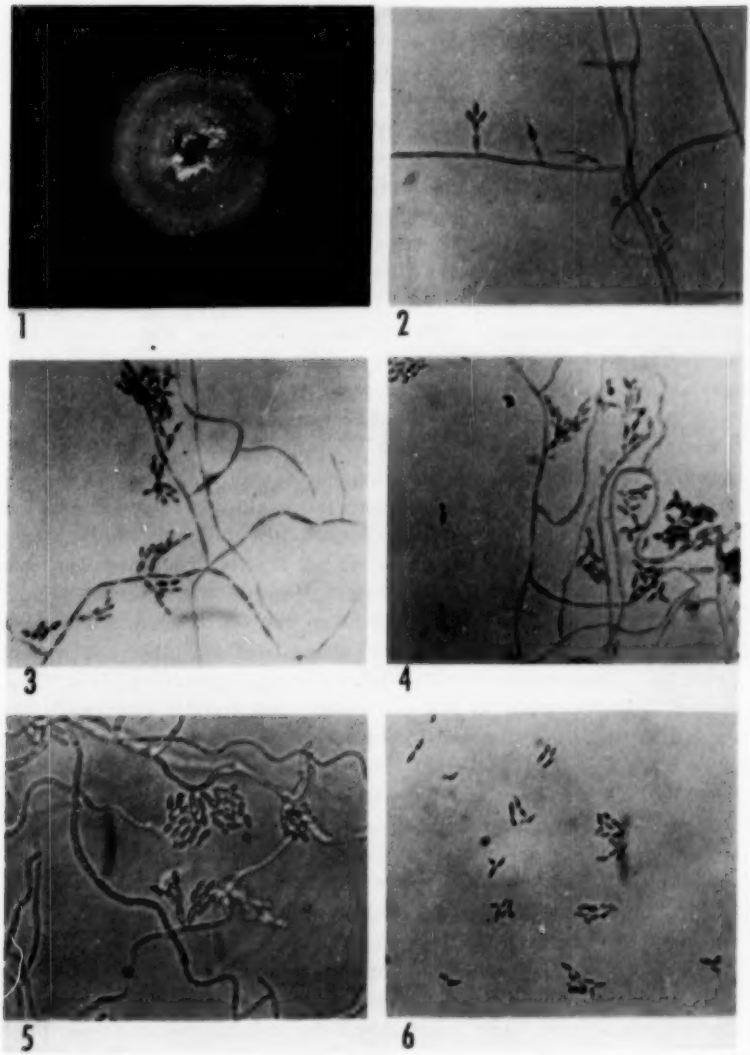
WEATHER DATA FOR TWO COLLECTION DATES *

Site	Date	Temp. °F	Wind direction	Wind velocity (mph)	Relative humidity
Atlanta	10–28	54	W	11	76%
Chamblee	2–17	52	WNW	14	58

* One P.M. each date. Data supplied by aerology office, U. S. Naval Air Station, Chamblee.

tricellular spores on branching conidiophores resembling those of the pionnotes of *Fusarium*. Upon transfer to potato dextrose agar slants, followed by incubation at room temperature, the resulting colonies attained a diameter of approximately 9 mm in 7 days, being similar in appearance to the originals and displaying concentric zonation. The colonial topography was low pyramidal-crateriform, the rim of the depression appearing irregular and fasciculate (FIG. 1). The reverse was concolorous. PDA slide cultures at this time displayed numerous branched conidiophores and a few 3-celled hyaline spores (FIGS. 3, 4). The aerial mycelium grew sparsely in a thin zone, while the submerged growth, bearing dense, complex conidiophores, was much heavier (FIG.

[1] Present address: Department of Bacteriology, Medical College of South Carolina, Charleston, S. C.



FIGS. 1-6. *Tricellula inaequalis:* colonial and microscopic morphology on potato dextrose agar. 1. Colony on agar plate. 2. Early stage in development of conidiophores, illustrating manner of budding. 3, 4. Aerial mycelium, well-developed conidiophores, and tricornate conidia. 5. Submerged mycelium, conidiophores and conidia. 6. Free conidia, 2- and 3-celled.

5). After 11 days the slant colonies were 18–20 mm in greatest diameter, light orange, moist, and glabrous, the crater rim being heaped and fasciculate and the peripheral 2–4 mm of the colony being flat, appressed, and paler in color. At the end of 30 days there had developed limited areas of sparse white, cottony, aerial hyphae.

Colonies on Sabouraud dextrose agar were similar in appearance generally to those on PDA and developed at about the same rate. However, radial grooves had appeared by the 11th day and the center of the colony was tufted, pale orange, and showed a white bloom, the remainder of the surface being covered with short, downy, aerial mycelium. By 30 days the colony had attained a diameter of 40 mm, limited by the confines of the substrate. The surface at this time was velvety and fasciculate, with deep radial grooves which formed depressions in the agar as seen on the concolorous reverse.

It was observed that litmus milk inoculated with a portion of mycelium formed a rennet curd in 3 to 5 days. This was followed by peptonization, the process becoming complete in about 10 days.

The mycelium is branched, septate and hyaline. Conidiophores are usually compound and appear to be formed by budding (FIG. 2), as are the spores arising therefrom, which are not readily distinguishable from fragments of the supporting structure. Many of the dehisced conidia comprise only two cells, usually in tandem, but characteristically a third cell, laterally placed and differing in shape from the others, is present (FIG. 6). The component cells, each measuring approximately $2 \times 5\ \mu$, appear to be separated by distinct walls. The conidia are readily detached, so that the two-celled forms may represent an immature stage.

While taxonomic studies of this organism were in progress, there appeared a paper by van Beverwijk (2) describing three new fungi, one of which, *Tricellula inaequalis*, appeared in almost all respects to be identical with the present isolates. Van Beverwijk described *T. inaequalis* as a new genus and species, the single isolate having been obtained "from an agar plate exposed for 15 minutes in an open square outside the University of Bergen (Norway)." She gives an excellent characterization of the manner of formation of the tricellular spores and their conidiophores, which accords very closely with that observed in the Atlanta isolates. Her paper should be consulted for this description and for those of the colonies on her media. The genus *Tricellula* is assigned by her to the sub-family Staurosporae of the Gloiosporae. A subculture of the Atlanta isolate was sent to Dr. van Beverwijk and a recent communication from her states that "It is in every respect identical with my isolate of this species."

It is intriguing that all three isolates of *T. inaequalis* thus far known have been collected from the air. Meteorological data presented with the Norwegian strain are as follows: "For a week there had been a layer of 10 cm of snow. The temperature was below 2° C, the sky was clear, no wind, low humidity." (Compare TABLE I.)

Transplants of the Atlanta strain have been deposited with the Centraal Bureau voor Schimmelcultures, the American Type Culture Collection, the London School of Hygiene and Tropical Medicine, and the Instituto Oswaldo Cruz.

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1. **Gordon, M. A.** Airborne Mold Flora of the Atlanta Area, 1953–54. In preparation.
2. **van Beverwijk, A. L.** Three new fungi: *Helicoon pluriseptatum* n. sp., *Papulaspora pulmonaria* n. sp., and *Tricellula inaequalis* n. gen., n. sp. Antonie van Leeuwenhoek **20**: 16 pp. 1954.

NOTES AND BRIEF ARTICLES

A note on Sirobasidium

The genus *Sirobasidium* was established by Lagerheim and Patouillard in 1892,[1] based upon collections from Ecuador. Two species were described, *S. albidium* and *S. sanguineum*. Later, three other species were described, namely, *S. Brefeldianum* Möll. Protobas. 165. 1895, from southern Brazil; *S. cerasi* Bourd. & Galz. Hym. Fr. I: 19. 1909, from France, and *S. magnum* Boed., Bull. Jard. Bot. Buitz. III. **13**: 267. 1934, from Borneo and Java.

The type of *S. cerasi* Bourd. & Galz. was examined by Boedijn, who found that "it not only proved to be no *Sirobasidium* but even its position in the basidiomycetae seems highly questionable."

The genus was first collected in North America by Coker [2] in 1920, who found *S. Brefeldianum* Möll. in North Carolina. In 1946, Olive [3] reported *S. sanguineum* from Maryland and again in 1951 [4] from Louisiana. The latter collection was not described and in view of the few records of *Sirobasidium* from North America, this note concerning a recent collection of *S. sanguineum* Lager. & Pat. from Louisiana is presented.

Fructifications forming buff-colored (Villalobos [5] 0, 12, 12° or Raw Sienna of Ridgway), soft-gelatinous, pustulate masses up to 4 mm high, 1 cm long and 5 mm broad, drying to form inconspicuous, resupinate reddish-brown patches (Villalobos SSO 3, 8° or approximately Burnt Umber of Ridgway). Basidia produced in short chains up to 4 cells, becoming variously septate, mostly 4-celled, sub-spherical to ovoid, (13.4–)14.2–16.6(–18) μ × (14.5–)15–17.5 μ. Basidiospores hyaline, fusiform, 1-celled, (14.2–)15.1–18.5(–24) μ × (5.4–)6.1–6.8(–7) μ, arising directly from the basidium and germinating by germ tube. Hyphae with clamp connections, 1.5–2.5 μ in diameter. Found on a dead branch

[1] Lagerheim, N. G. v. and N. Patouillard. *Sirobasidium*, nouveau genre d'Hyménomycètes Hétérobasidies. Jour. de Bot. **6**: 465–469. 1892.

[2] Coker, W. C. Notes on the lower basidiomycetes of North Carolina. Jour. Elisha Mitchell Soc. **35**: 113–182. 1920.

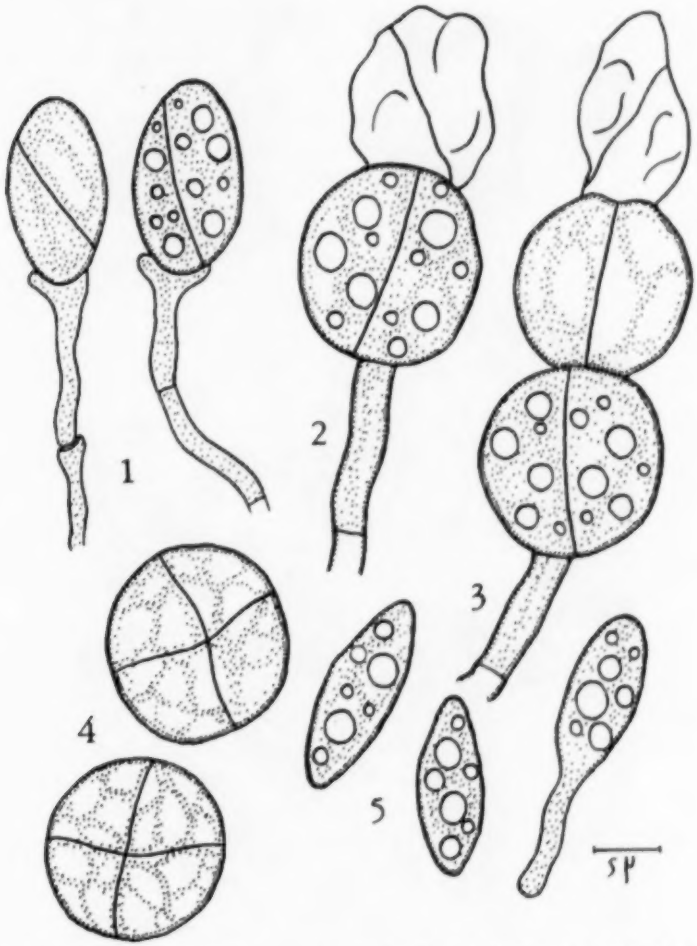
[3] Olive, L. S. Some taxonomic notes on the higher fungi. Mycologia **38**: 534–547. 1946.

[4] Olive, L. S. Taxonomic notes on Louisiana fungi. III. Additions to the Tremellales. Mycologia **43**: 677–690. 1951.

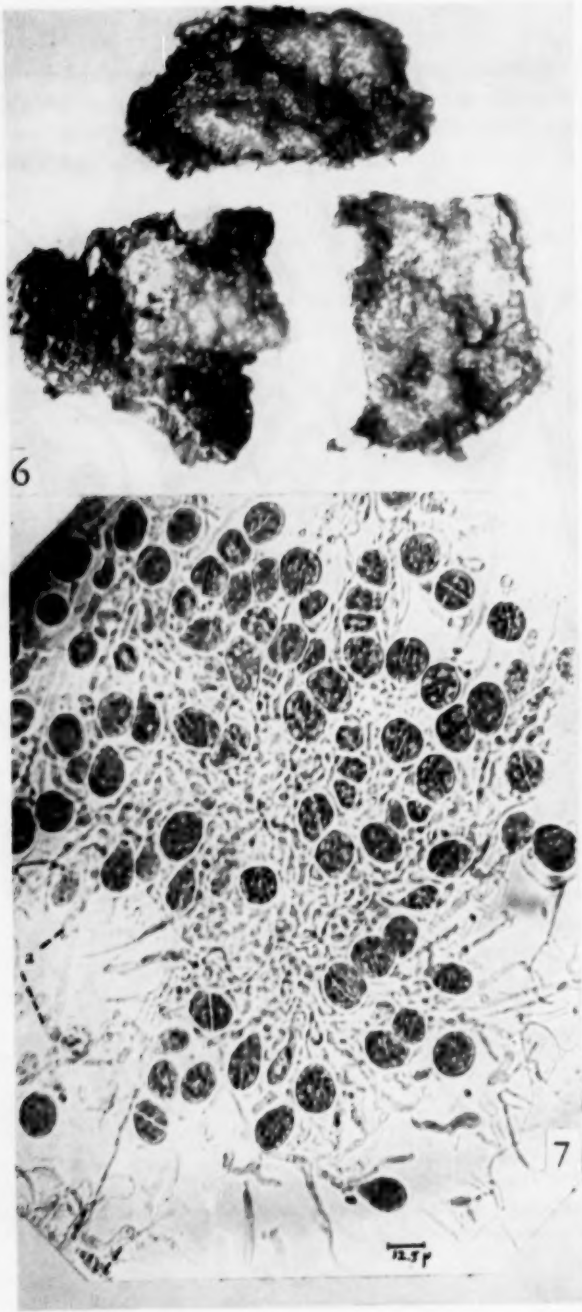
[5] Villalobos, C. y J. Atlas de los colores. Buenos Aires. 1947.

(about a meter long) of *Fraxinus nigra* in a swamp about 5 miles south of the University campus, Baton Rouge, La., November 17, 1955.

A conspicuous difference between the Louisiana collection and the original description of *S. sanguineum* by Lagerheim and Patouillard [1]



FIGS. 1-5. *Sirobasidium sanguineum*. 1. Two basidia with oblique septa. 2. Group of 2 basidia, apical basidium collapsed. 3. Chain of 3 basidia, apical basidium collapsed. 4. Two detached cruciately septate basidia. 5. Group of 3 basidiospores, one germinating by germ tube.



FIGS. 6–7.

was seen in the lack of red pigment in any part of the former. According to Lagerheim and Patouillard, the fruiting body is blood-red, hyphal walls are reddish and basidia slightly reddish. Martin [6] observed a similar loss of color in an Australian collection of *S. sanguineum* and noted that "its pallid appearance may well be due to the fact that the color has been washed out, as not infrequently happens in the case of tremellaceous fungi with red or yellow tints."

Collections were placed in a moist chamber and after 30 hours many basidiospores were found to be germinating by germ tubes. Observations were continued at intervals for 4 days, during which time no basidiospores were seen to germinate by repetition.

A key to the 4 species of *Sirobasidium* recognized follows.

1. Basidia predominantly 4-celled.....2
1. Basidia predominantly 2-celled.....3
2. Basidiospores 24–26 × 6–10 μ.....*S. albidum*
2. Basidiopsores 17–20 × 6–8 μ.....*S. sanguineum*
3. Fructification minute, about 3 mm or less.....*S. Brefeldianum*
3. Fructification large, up to 7 cm.....*S. magnum*

—BERNARD LOWY, Department of Botany, Bacteriology and Plant Pathology, Louisiana State University, Baton Rouge, La.

AN UNUSUAL PHALLOID FROM MASSACHUSETTS

Last summer we received a phalloid of somewhat unusual occurrence. It was collected by Mrs. Sybil Curtis of the Boston Mycological Club daily from August 23rd to September 2nd close to a cluster of *Mutinus caninus* (Huds. ex Pers.) Fr. on a huge pile of leaves accumulated from several years of leaf-raking, in Millbury, Mass. It appears to be *Pseudocolus Shellenbergiae* (Sumstine) Johnson—to use a convenient epithet and to avoid considerations of synonymy or taxonomy. —WALTER H. SNELL AND ESTHER A. DICK, Brown University, Providence, R. I.

[6] Martin, G. W. Some Australian Heterobasidiomycetes. Mycologia 28: 214–221. 1936.

FIGS. 6–7. *Sirobasidium sanguineum*. 6. In expanded (wet) condition, × 2.5. 7. Photomicrograph showing variously septate basidia occurring singly and in short chains; clamp connections at (a) and a basidiospore at (b).

THE DEVELOPMENT OF BOUDIERA AREOLATA ON SOIL IN THE LABORATORY

For the benefit of persons interested in certain operculate cup-fungi I am reporting a recent discovery.

In September of 1955 I collected pint jars of woodland soils from twelve states from Wisconsin to New Mexico and east to New York City. Each sample was placed in a Pyrex dish 9 cm wide and 5 cm deep and flooded with sterile distilled water. When the samples were saturated, the excess water was poured off and a Petri dish lid sealed over the dish by means of vaseline. The dishes were left at room temperature in normal light from October 7 to November 15.

On one sample, ascocarps of *Boudiera areolata* Cooke & Phill. first appeared on November 2 and more developed during the following ten days. The apothecia apparently are angiocarpic and at first are elongated to subglobose. In three or four days the mature (?) apothecia are formed. The fungus was identified as *B. areolata*, though the apothecia are 470–700 μ wide as compared with the recorded 2–2.5 mm, the spores become pale violet brown rather than the brown recorded by Saccardo and the paraphyses remain colorless rather than become brown. These differences may, of course, be due to failure of the fungus to develop to full maturity. Specimens are deposited at the New York Botanical Garden Cryptogamic Herbarium. The sample on which this fungus developed was a mixture of soil and leaf-mold collected in a maple-oak-witch hazel woods near Platteville, Wisconsin, on September 12, 1955.—JOHN B. ROUTIEN, Dept. of Biochemical Research, Chas. Pfizer & Co., Inc., 630 Flushing Avenue, Brooklyn 6, New York.

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